AN ABSTRACT OF THE THESIS OF

Susan Leers Sucheta for the degree of Doctor of Philosophy in Animal Science presented on May 13, 1993. Title: Secretion of Luteinizing Hormone in Response to Exogenous Melatonin in Postpartum Beef Cows and Ovariectomized Beef Heifers.

Abstract approved: Redacted for Privacy

Fredrick Stormshak

Two experiments were conducted to investigate the effect of exogenous melatonin (MLT) on LH secretion in beef cows and heifers. **Exp. 1**. Cows received none (C; n=5) or two (n=7) MLT implants (250 mg; s.c.), one in each ear, the day after calving. Blood was collected once weekly (1000 hr) and at 2400 hr on day 27 postpartum (pp) for MLT analysis and every 15 min for 4 hr on days 28 (day of calf removal; CR), 29 and 31 pp for LH analysis. After the sampling period on day 31 each cow was injected with GnRH (100 $\mu$g, i.v.) and sampling was continued at 15 min intervals for 2.5 hr. Weekly daytime ($P<.01$) but not midnight ($P>.05$) concentrations of MLT were greater in MLT compared with C cows. Secretion of LH over the 3 days and in response to GnRH did not differ between groups ($P>.05$); however, daily LH secretion was more variable among C than MLT cows ($P<.01$). Fewer MLT cows (0/7) returned to estrus within 15 days of CR

compared with C cows (3/5; $P<.05$). **Exp. 2.** Heifers ovariectomized (OVX) for 6 mo received none (C; n=4) or one (n=4) MLT implant (1 g, s.c.) in the neck. On day 29 after implant insertion, heifers were injected with estradiol-17$\beta$ ($E_2$; 1.5 mg, i.m.). Blood was collected once weekly for MLT analysis, and after $E_2$, every 45 min for 24 hr for LH analysis. Serum MLT concentrations were greater in treated vs C OVX heifers ($P<.01$); however, LH secretion was not affected by treatment ($P>.05$). Results of this study indicate that reduced variability in LH levels among MLT-treated cows was associated with the absence of estrus and ovulation after CR, suggesting that MLT may have prevented these events through subtle modulation of LH secretion. Treatment with MLT failed to affect $E_2$-induced LH secretion in OVX heifers.

# Secretion of Luteinizing Hormone in Response to Exogenous Melatonin in Postpartum Beef Cows and Ovariectomized Beef Heifers

by

Susan Leers Sucheta

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed May 13, 1993

Commencement June 1994

APPROVED:

Redacted for Privacy

Professor of Animal Sciences and Biochemistry and Biophysics in charge of major

Redacted for Privacy

Professor of Animal Sciences in charge of major

Redacted for Privacy

Head of Department of Animal Sciences

Redacted for Privacy

Dean of Graduate School

Date thesis is presented May 13, 1993

Typed by Susan Leers Sucheta

# DEDICATION

This thesis is dedicated to the memory of my brother William Leers and nephew Jaime Walker. I know they would have achieved similar or greater goals had they lived to reach their full potential.

Through every rift of discovery some seeming anomaly drops out of the darkness, and falls, as a golden link, into the great chain of order.

--Edwin Hubbel Chapin

# ACKNOWLEDGMENTS

I gratefully thank Dr. Martin Vavra, superintendent of the Eastern Oregon Agricultural Research Center, for providing my research assistantship and many of the cows used in my research efforts under the direction of Dr. Harley Turner and Dr. Fredrick Stormshak. Additional thanks are expressed to the research center staff for their assistance during my 6 week "visit" to inject and bleed postpartum cows.

I would like to thank my major advisors, Dr. Turner and Dr. Stormshak for their guidance and support during my research and preparation of this dissertation. I also express thanks to my graduate committee: Dr. Warren Kronstad, Dr. Frank Moore and Dr. Henry Schaup for their participation in my degree program and discussion of my research results. Special thanks are also due Dr. Ken Rowe for performing many of the statistical analyses presented in this work and Dr. Prabir Chakraborty for his advise regarding iodination of LH and donation of the primary (PKC-242) and secondary (PKC-pool C) antibodies used in the LH assay. I would also like to thank Bev Vanderplaat for making all the arrangements for my thesis defense.

Because the experiments described in this thesis required a great deal of animal handling and hours of blood sampling, it would have been extremely difficult if not impossible to conduct them without the assistance of a number of people. I extend my heartfelt thanks to Dr. OV Slayden for always dropping his own work to assist me with the cows and calves, for not telling me to get lost after the first calf left indelible hoof prints on his insteps and for staying late on those nights when I needed to collect midnight blood samples. I also wish to express my gratitude to Marvin Martin for teaching me how to work cattle without getting completely stressed-out; he is an expert. Special thanks also go to Patrice Prater for her assistance during the 24 hour bleed of the OVX heifers; especially for

appearing out of nowhere the second morning when the fatigue factor was in high gear. I also thank Teri Martin, Anna Cortell, Ligaya Tumbelaka, Tom Dill, and Kyle Orwig for their help with blood collection during different experiments. I am also grateful to Kay, Rainier and Dan at Radiation Safety for their friendship and for making routine thyroid monitoring an enjoyable activity. Additionally, I would like to thank Dr. Stormshak for allowing me the opportunity to veer from the straight and narrow confines of previous research in order to explore less well established paths. Although the experimental outcome was not always positive, the learning experience was invaluable.

I also gratefully acknowledge Dr. D. J. Bolt, USDA Animal Hormone Program, Beltsville Agricultural Research Center, MD, for the donation of purified bLH (USDA-bLH-B-5, AFP 5500), the National Institutes of Health, National Hormone and Pituitary Program for the donation of biological grade NIH-bLH-B-10 and Dr. Myron Brown and Dr. C. M. Tucker, Sanofi Animal Health, Overland Park, KS, for their donations of GnRH.

Finally, I would like to thank those members of my family that believed more in my capabilities as a scientist than I did and provided never-ending love and support during my program. I am grateful to my parents Edward and Sarah Leers for making me believe that anything is possible if you work at it hard enough and for encouraging me to extend my graduate career beyond that of the M.S. I also wish to express my gratitude to Tom and Rozella Leers; their assistance and support made my M.S. degree possible and provided the foundation for my Ph.D. studies at O.S.U. To my husband, Dr. Artur Sucheta, I express enormous amounts of appreciation for his loving support over the course of my graduate studies. In addition, he provided excellent technical advice on everything from lab procedures to computer graphics and was never too busy to patiently explain mathematical or chemical concepts that I found difficult to understand. You are a wonderful teacher.

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## INTRODUCTION

Efficient production of beef cattle requires that cows wean one calf per year. Therefore, it is critical that cows return to estrus and are successfully bred by 90 days postpartum. Cows that are not pregnant by this time, represent an economic loss to the producer. Regulation of the postpartum period in cows is complex and involves many hormones that ultimately affect the secretion of luteinizing hormone (LH). Environmental factors such as suckling and nutrition have been identified as factors that affect the interval from calving to first estrus. However, the specific mechanisms through which these factors regulate the length of anestrous period are unclear. Even more poorly understood is the effect of season or melatonin on the secretion of LH during postpartum anestrus.

Melatonin secreted by the pineal gland has been shown to mediate the photoperiodic control of reproduction in seasonal breeders. Seasonally breeding animals are those that reproduce (mate) during specific seasons of the year, usually in the autumn (fall) or spring, and are commonly referred to as "short"- or "long"- day breeders in reference to the prevailing photoperiod (decreasing or increasing duration of daylight) associated with initiation of the breeding period. In most mammals, melatonin is secreted in a diurnal rhythm with maximal systemic concentrations occurring during the dark period. Cattle exhibit a marked rise in both plasma and cerebrospinal concentrations of melatonin at the onset of darkness, which is sustained throughout the dark period (Hedlund *et al.*, 1977; Kennaway *et al.*, 1977; Berthelot *et al.*, 1990). The pineal gland is believed to mediate

photoperiodic control of ovine reproduction through modification of the hypothalamic gonadotropin releasing hormone (GnRH) pulse generator to ultimately alter the pattern of LH release (Bittman *et al.*, 1985). High affinity melatonin receptors have been identified in bovine medial basal hypothalamic membrane preparations (Cardinali *et al.*, 1979). Melatonin implants increased daytime serum concentrations of melatonin and delayed the onset of estrus and ovulation in anestrous Shorthorn beef cows (Sharpe *et al.*, 1986). These latter data suggest that melatonin may influence the interval from parturition to first estrus in beef cows. Scant research has been conducted to investigate the effect of melatonin on LH secretion during the anestrous period of beef cattle.

This dissertation contains a review of contemporary scientific literature on known and proposed factors that regulate the estrous cycle and postpartum interval of heifers and cows, and presents the results of two novel experiments that examine the effects of exogenous melatonin, administered as a subcutaneous silastic implant, on LH secretion of postpartum cows subjected to short-term calf removal and in ovariectomized beef heifers.

# REVIEW OF THE LITERATURE

## Estrous Cycle of the Cow

Regulation of the bovine estrous cycle involves participation of the hypothalamic-hypophysial (pituitary) axis, ovary and uterus. Ovarian and endocrine events that occur during the estrous cycle have been extensively reviewed for cattle, sheep and swine (Hansel and Echternkamp, 1972; Hansel and Convey, 1983) and the following is a brief overview of the main events that transpire over the course of a typical bovine estrous cycle. It is generally accepted that the average duration of the estrous cycle of the cow is 21 days. The actual period in which the cow is receptive to mating is termed estrus and is characterized by a willingness to stand when mounted by a bull or other cows. Rising levels of estradiol-17$\beta$ (hereafter referred to as estradiol) precede the onset of estrus and stimulate increased secretion of pituitary LH and follicle stimulating hormone (FSH). Concentration of LH in blood peaks 24 to 32 hr after the onset of estrus, which usually lasts about 18 hr, and triggers ovulation. Estradiol and FSH levels also peak concomitantly with LH but do not stimulate ovulation directly. Following ovulation, granulosa and theca cells lining the ruptured follicle luteinize to form the corpus luteum (CL) and begin to secrete progesterone in response to LH. Growth of the CL occurs rapidly from days 3 to 12 of the cycle and then reaches a plateau. At this time, concentrations of estradiol and gonadotropin are low, and with the exception of several transient increases in these hormones during the luteal phase, remain low prior to luteal regression. Corpus luteum growth and progesterone secretion parallel one another and are maximal midcycle (days 9 to 12). Progesterone

secretion remains elevated until late in the cycle when the CL begins to regress (days 16 to 19) under the influence of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) secreted by the uterus. As the CL regresses, progesterone secretion declines sharply followed by secretion of increasing levels of estradiol, LH and FSH as the cow returns to estrus and a new cycle begins.

Folliculogenesis, ovulation, and developmental and functional aspects of the corpus luteum during the estrous cycle of the nonpregnant cow will be discussed in detail in the following sections. In addition, initiation of estrous cycles in the postpartum cow will also be addressed.

**Folliculogenesis**

Control of follicular growth and development in the bovine is a broad area of research that has been extensively reviewed in the past 10 years (Richards, 1980; Spicer and Echternkamp, 1986; Ireland, 1987; Fortune *et al.*, 1991). By the time of birth female mammals have acquired all of the oogonia that they will have during their lifetime. Early in development gonadal ridges are formed in the undifferentiated fetus. Primordial germ cells from the embryonic hindgut migrate to the developing ovary where they differentiate into oogonia. Oogonia increase in number through mitotic division and subsequently develop into larger cells called primary oocytes. Prior to birth, mitotic activity ceases in these cells whereupon they enter prophase of the first meiotic division and remain arrested in this stage until they degenerate (undergo atresia) or are released by ovulation (Gondos, 1978; Byskov and Høyer, 1988). The embryonic origin of granulosa cells is still unclear, but it appears that they arise from the *rete ovarii* (Byskov, 1974), not ovarian surface epithelium (Byskov *et al.*, 1977), and organize around the primary oocytes resulting in a

pool of primordial (nongrowing) follicles. Follicular growth is stimulated prenatally in large mammals (cow, sheep, monkey, human) and begins with enlargement of the primary oocyte, proliferation of surrounding granulosa cells and organization and formation of theca cell layers external to the basement membrane surrounding the granulosa cells (Peters, 1978).

An excellent illustration and description of the classification of ovarian follicles during their growth and development (Erickson *et al.*, 1985) depicts 5 classes of follicles: **primordial**, **primary**, **secondary**, **early tertiary** and **Graafian**. Primordial follicles are characterized by a single layer of granulosa precursor cells that surround the oocyte and are circumscribed by the basal lamina (basement membrane) separating the follicle from the ovarian stroma. Progression to the primary follicle involves the formation of the zona pellucida (glycoprotein band) that separates one or more layers of granulosa cells layer from the oocyte. Secondary follicles have multiple layers of granulosa cells and are, therefore, larger in diameter than primary follicles. In addition, these follicles are primarily characterized by the appearance of the presumptive theca layer surrounding the basal lamina. Early tertiary follicles can be distinguished from secondary follicles by the formation of an antrum within the granulosa cell layer and differentiation of the theca into interna and externa layers that are invaded by blood vessels. The most distinctive characteristic of the Graafian (preovulatory) follicle is the presence of the cumulus oophorus (hillock of granulosa cells), which forms as the antrum expands and displaces the oocyte from the center of the follicle. The cumulus oophorus is comprised of both the granulosa cells forming the hillock as well as the corona radiata cells that immediately surround the oocyte. In addition, the theca interna and externa are more clearly defined and the antrum is completely formed and filled with follicular fluid. Therefore, according to this classification, preantral follicles

would include both primary and secondary follicles whereas antral follicles would include tertiary and Graafian follicles.

***Number and Distribution of Follicles***

Development and senescence of the postnatal bovine ovary was described over 25 years ago by Erickson (1966). Ovaries from either cycling, pregnant or anestrous Hereford cows of various ages (range:birth to 20 years) were removed at slaughter and total numbers of primordial and growing follicles were estimated per pair of ovaries. Follicles were categorized as either **primordial** (oocyte encompassed by a single layer of granulosa cells), **growing** (oocyte encompassed by two or more layers of granulosa cells but without a fully formed antrum), or **antral** (follicle with fully formed antrum). Further, primordial follicles counted were also graded as normal (chromatin in dictyate state) or abnormal (chromatin agglomerated) while antral follicles were graded normal or atretic (degenerative changes or absence of granulosa cells). Numbers of primordial follicles remained stable from birth until 4 to 6 years of age (mean = 133,000 follicles) and then declined until approximately 3000 remained in the ovaries of 15 to 20 year-old-cows. Quality of germ cells (oocytes) in primordial follicles was high (mean = 81.5% normal) in ovaries up to 180 days of age and then declined rapidly whereupon nearly all follicles in ovaries from cows 4 years of age and older were in a degenerative state (range = 71 to 100% abnormal). Numbers of growing follicles were observed to increase rapidly between 50 and 80 days of age (increase from 93 to 204 follicles), followed by a gradual increase up to 120 days of age (mean = 227 follicles) with little change from 120 days to 4 to 6 years of age. Numbers of growing follicles began to decline from age 7 and reached a postpubertal low (mean = 72 follicles) in ovaries from 15 to 20 year-old-cows. Although numbers of antral follicles were observed

to increase coincidentally with the pool of growing follicles, fewer numbers of antral follicles were observed at each age group. Further, the antral follicle population appeared to be divided equally between normal and atretic states in all age groups. It is clear, from the data presented above, that follicular growth and development occur continuously throughout the estrous cycle and life span of the cow. In addition, as the animal ages, populations of normal primordial, growing preantral and antral follicles decrease with age providing fewer numbers of potential normal, nonatretic follicles to participate in the selection of the ovulatory follicle.

Development of antral follicles has been followed over the estrous cycle of beef heifers (Ireland *et al.*, 1979). Because precise day of the cycle was not known, stage of the cycle (days 1 to 4, 5 to 10, 11 to 17, 18 to 20) was estimated by appearance of the CL within 30 min of slaughter. Follicles protruding to the surface of each pair of ovaries were counted. Follicular size was determined by measurement of external diameter and volume of aspirated follicular fluid (FF). Follicles were categorized as **small** (<3 mm; 5 to 100 $\mu$l FF), **medium** (3 to 9 mm; 101 to 400 $\mu$l FF) and **large** (>10mm; >400 $\mu$l FF). Mean total numbers of follicles on the surface of the ovary were greater late in the cycle (46 $\pm$ 5; days 18 to 20) as compared with early in the cycle (38 $\pm$ 3; days 1 to 4). All heifers had follicles in the small range during each stage of the cycle (mean = 40 $\pm$ 2), whereas, the percentage of heifers having follicles in the medium range did not differ between stages of the cycle and averaged 38 percent. A greater percentage of heifers exhibited large follicles from days 5 to 10 (88%) and 18 to 20 (73%) compared with those on days 1 to 4 (30%) of the cycle. No heifers had medium and large follicles present on either ovary during days 1 to 4 of the cycle. The occurrence of at least one medium and large follicle on the same

ovary was rarely observed except during days 5 to 10 of the estrous cycle. In fact, no medium and large follicles coexisted on the same ovary early (days 1 to 4) or late (days 18 to 20) during the estrous cycle. Others have reported similar observations in cattle (Dufour *et al.*, 1972; Matton *et al.*, 1981). Collectively, these data demonstrate that small, medium and large follicles are present during all stages of the bovine estrous cycle and that large follicles appear more frequently after day 4 of the cycle. In contrast, the occurrence of medium-sized follicles does not seem to vary with stage of the estrous cycle.

***Follicular Steroid Biosynthesis***

Intrafollicular sites of steroid biosynthesis, and their regulation, have been of great interest since the beginning of this century when estrogen was first identified in follicular fluid (Allen and Doisy, 1927). It has been known for over 50 years that synthesis of steroids by theca and granulosa cells is regulated by FSH and LH (Fevold, 1941). The ability of these two cell types to respond to FSH and LH is dependent not only upon the number and type of receptor present but on the concentration of gonadotropin in the serum as well. Granulosa cells of both preantral and antral follicles possess receptors for FSH, however, only granulosa cells of large preovulatory follicles have receptors for LH. Theca cells, on the other hand, gain LH receptors while in the preantral stage but never possess FSH receptors (Richards, 1980). Studies of rat granulosa cells *in vivo* demonstrate that FSH can up-regulate its own receptor (increased number) in the cell membrane; however, *in vitro* studies indicate that continuous exposure of granulosa cells to FSH stimulation results in down-regulation of the receptor (reduced number) resulting in decreased FSH responsiveness. Appearance of granulosa LH receptors is induced by FSH and requires the continued presence of FSH for their maintenance. As was the case with FSH, exposure of

granulosa cells to excessive LH results in down-regulation of its receptor and decreased responsiveness to this gonadotropin (Hsueh, 1984). Because receptors for both FSH and LH are present in preantral granulosa and theca cells, respectively, follicles are capable of responding to these gonadotropins at a very early stage of development.

Hormonal regulation of granulosa cell steroid biosynthesis has been reviewed (Hsueh *et al.*, 1984). Upon stimulation with FSH, granulosa cells from preantral and antral follicles (both *in vivo* and *in vitro*) are able to synthesize progesterone *de novo* from cholesterol because they contain the necessary side-chain cleavage (cholesterol → pregnenolone) and 3$\beta$-hydroxysteroid dehydrogenase (3$\beta$-HSD; pregnenolone → progesterone) enzymes. The cytochrome P-450 side-chain cleavage (SCC) enzyme complex resides in the inner membrane of the mitochondrion and therefore the conversion of cholesterol to pregnenolone occurs in this organelle. Further, this conversion is the rate-limiting step in progestin biosynthesis. Once formed, pregnenolone readily diffuses from the mitochondrion and is subsequently converted to progesterone through the action of 3$\beta$-HSD located in the smooth endoplasmic reticulum. Induction and activity of P-450 SCC and 3$\beta$-HSD enzymes in preantral and antral follicles is regulated by FSH. Once granulosa cells acquire LH receptors, through FSH induction, LH invokes progesterone production in large preovulatory follicles by increasing cytochrome P-450 SCC and 3$\beta$-HSD activity. Progesterone cannot be further metabolized to androgens in these cells because they lack the 17$\alpha$-hydroxylase and 17-20 desmolase enzymes required to convert progestins to androgens. Although granulosa cells cannot synthesize androgens directly, they do possess the aromatase enzymes necessary for estrogen production from androgen precursors. Because androgens such as testosterone and androstenedione are precursors to estrogen production, granulosa cells must rely on another

cellular source for these steroids in order to synthesize estradiol. It should be noted, however, that progesterone production by granulosa cells is relatively low compared with that of estrogens, except immediately prior to and during the LH surge when progesterone production seems to increase (Ireland and Roche, 1983b).

Structure and function of ovarian androgen producing cells has been reviewed (Erickson *et al.*, 1985). Convincing evidence that the ovary produces androgen was reported as early as 1937. Ovaries transplanted to the ears of male castrated mice returned the atrophic secondary sex glands in these rodents to a normal state (Hill, 1937). Subsequent transplant experiments performed by Falck and coworkers (1962) localized ovarian androgen production, in rats, to the theca interna of follicles. Prior to differentiation, precursor theca cells contain LH receptors but lack cytochrome P-450 SCC, 17$\alpha$-hydroxylase (progesterone $\rightarrow$ 17$\alpha$-hydroxy progesterone), and 17-20 lyase (17-hydroxyprogesterone $\rightarrow$ androstenedione) enzymes required for androgen synthesis. Stimulation with LH results in cytodifferentiation of these cells and induction and stimulation of the enzymes needed for androgen biosynthesis (Erickson *et al.*, 1985).

Briefly, LH and FSH exert their actions in granulosa and theca cells, by binding to specific receptors located in the plasma membrane. Binding of hormone to receptor activates adenylate cyclase and ATP is converted to cAMP. This cyclic nucleotide activates protein kinase A and results in the phosphorylation of specific intracellular proteins, which in turn stimulate the synthesis of the cell specific enzymes necessary for steroidogenesis in these two cell types. A more detailed account of the mechanism of action of protein hormones, such as LH and FSH, on target cells is presented in a later section of this review.

Current scientific dogma recognizes the **two-cell, two-gonadotropin theory** of follicular estrogen production as one of the most important principles of ovarian physiology (Leung and Armstrong, 1980; Richards, 1980; Erickson *et al.*, 1985; Spicer and Echternkamp, 1986; Amsterdam and Rotmensch, 1987; Ireland, 1987). According to this model, both theca interna and granulosa cells, as well as, LH and FSH are required for intrafollicular estrogen synthesis. More specifically, LH stimulates the production of androgen from cholesterol in the theca interna and due to their lipophilic nature, androgens diffuse freely across the basement membrane into granulosa cells where FSH and LH stimulate their aromatization to estrogens, which are then released into the ovarian vein or follicular fluid. In addition to *de novo* synthesis of androgen by theca cells, granulosa-derived pregnenolone, synthesized in response to LH, may diffuse across the basement membrane into theca cells where it is metabolized to androgen to provide additional estrogen precursor for the granulosa cells (Fortune, 1986).

Experiments conducted to determine the role of granulosa and theca cells in bovine follicular steroid biosynthesis support the two gonadotropin, two cell hypothesis. Theca interna of healthy, nonatretic follicles is the source of ovarian androgen in the bovine ovary and the primary androgen produced by this tissue is androstenedione (McNatty *et al.*, 1984b). These investigators used an *in vitro* perfusion system to determine that the majority of total steroid produced by isolated theca cells was androstenedione (82%) as compared to the production of progesterone (1%), testosterone (15%), and estradiol (2%). In contrast, isolated granulosa cells produced only progesterone (79%) and estradiol (21%). Further, when these granulosa cells were provided saturating concentrations of androstenedione, they generated greater than 90% of the total quantity of estradiol produced by both tissues in

isolation. These data suggest that, in the bovine follicle, granulosa and theca interna cells are the primary sites of estradiol and androstenedione synthesis, respectively. It should be noted, however, that although the two cell, two gonadotropin model is useful for describing follicular estradiol synthesis in the rat, pig, cow, ewe and rabbit, the theca interna may be the primary site of estrogen synthesis during some stages of follicular development in the human and horse (Hsueh *et al.*, 1984).

***Recruitment and Selection of the Dominant Follicle***

Goodman and Hodgen (1983) suggested the use of the terms **recruitment, selection** and **dominance** when describing the processes that lead to the development of ovulatory follicles. Follicle recruitment is a gonadotropin-dependent event in which a cohort of follicles gains the ability to respond to gonadotropins and requires gonadotropins for continued growth. In each follicular wave, recruited follicles undergo a selection process whereby the preovulatory follicle(s) is chosen to escape atresia and becomes dominant and continues development toward ovulation while the others regress. The dominant follicle will ovulate only if luteal regression has been initiated during that wave.

Regulation of follicular recruitment is not fully understood and has received less attention than the process of selection of the dominant follicle. Most research in this area to date has relied on the use of the hypophysectomized rat as the experimental model. Folliculogenesis, in the rat, is dependent on FSH because short-term hypophysectomy or short-term reduction of FSH inhibits the development of preantral and antral follicles and administration of FSH restores antral follicle development (Ireland, 1987). Further, in rats, it appears that the preovulatory surge of FSH, as well as LH, stimulates recruitment of the

next wave of follicles from which the ovulatory follicles of the next cycle will be selected (Richards, 1980).

The processes of selection and dominance have been exceptionally well studied in cattle and are associated with temporal alterations in ovarian venous and systemic estradiol concentration. Therefore, fluctuations in estradiol secretion can be used as an endocrine marker for these two events (Ireland and Roche, 1987). These authors suggest that symmetrical ovarian production of estradiol occurs during the selection process, whereas, asymmetrical production of estradiol occurs during the dominance phase. Large non-ovulatory and preovulatory dominant follicles contain higher concentrations of estradiol in follicular fluid than both progesterone and androgens (estrogen-active) and account for the bulk of estradiol found in ovarian venous blood. Slightly smaller subordinate follicles usually contain greater concentrations of progesterone or androgens than estradiol (estradiol-inactive) and are classified as atretic. Ovarian venous and peripheral blood levels of estrogen rise concomitantly with each follicular wave of selection during the estrous cycle and are thought to initiate the preovulatory surge of LH and behavioral estrus in cattle (Ireland and Roche, 1987).

Nearly 35 years ago, Rajakowski (1960) suggested that follicular development in cattle occurred in "waves". Using serially sectioned ovaries from heifers sacrificed on specific days of the cycle, he suggested that the first wave of development began early in the cycle and ended at midcycle with development of one large ovulatory follicle that eventually became atretic. The second wave began just after midcycle and culminated with the development of an ovulatory follicle.

A more advanced approach for the study of follicular dynamics *in vivo* was developed in the early 1970's and relied on the use of laparoscopy to follow the development of specific follicles marked with India ink. Results of two such experiments in cattle, (Dufour *et al.*, 1972; Matton *et al.*, 1981) support the concept that follicular development in heifers occurs in two waves. In both of these studies one follicle grew to be the largest by midcycle and regressed by day 18, whereas, the largest follicle on day 18 of the cycle became the ovulatory follicle.

More recently, the use of ultrasonography to examine follicular growth and development in the bovine ovary was described (Pierson and Ginther, 1984). Using ultrasound to monitor the number and sizes of follicles on the ovaries during the estrous cycle of heifers (Pierson and Ginther, 1987), these investigators suggested that two ovulatory-sized follicles develop during the cycle and that the ovulatory follicle is selected only 3-4 days prior to ovulation (Pierson and Ginther, 1988). Again these data are supportive of the concept that bovine follicles develop in two distinct waves during an estrous cycle and provide further evidence that the ovulatory follicle is not selected until very late in the cycle (days 17 to 18).

Also using ultrasonography, Sirois and Fortune (1988) reported that follicle development in heifers occurred in 3 waves in the majority of their heifers (75%) and in two waves in the remainder of the herd. Each wave was characterized by the concurrent appearance of a cohort of 3 to 6 follicles, greater or equal to 5 mm in diameter, in which one follicle became larger than the others (dominant) and continued to grow while the smaller follicles in the cohort began to regress. Individual waves began, on average, on days 2, 9 and 16 and culminated in ovulation of the dominant follicle of the third wave.

Others have reported the occurrence of three waves of follicular development in cattle (Savio *et al.*, 1988). In a subsequent study, Lavoir and Fortune (1990) explored the relationship between **morphological** (the largest follicle on a pair of ovaries) and **functional** (ability to suppress the growth of other follicles) dominance in non-ovulatory follicles. The criterion for functional dominance was defined as the ability of a follicle in the first wave of development to ovulate in response to a luteolytic injection of $PGF_{2\alpha}$ administered at various stages of growth (active growth, early plateau and late plateau) of the morphologically dominant follicle. These researchers reported that when $PGF_{2\alpha}$ was administered during the active growth phase of the dominant follicle, morphological dominance coincided with functional dominance as the largest follicle present usually ovulated. In contrast, a follicle from the second wave was recruited to ovulate when $PGF_{2\alpha}$ was administered during the late plateau phase of growth of the dominant follicle. In addition, when $PGF_{2\alpha}$ was administered during the early plateau phase of the dominant follicle, the morphologically dominant follicle did not always ovulate and resulted in the ovulation of a new follicle of the first wave that had already been observed at the time of $PGF_{2\alpha}$ injection. These data suggest that size alone is not a good indicator of functional dominance, because during the early plateau phase of growth the morphologically dominant follicle appears to be in transition between the functional dominance observed during active growth and non-dominance observed during the late plateau stage of growth.

Previous studies conducted to examine follicular development in cattle (Matton *et al,.*, 1981; Staigmiller and England, 1982) suggested that a single large follicle was able to inhibit the development of smaller follicles. In addition, bovine follicular fluid (FF) was reported to contain a non-steroidal factor that could inhibit follicular growth in heifers

(Miller *et al.*, 1979). The suppressive effect of the dominant follicle on the growth of subordinate follicles was demonstrated in dairy heifers treated with progesterone-releasing devices to artificially lengthen the estrous cycle. Heifers exhibiting only 2 or 3 waves over the 30 day cycle had prolonged periods of follicular development during the final wave which were characterized by larger than normal growth of the dominant follicle and complete suppression of the development of other follicles (Sirois and Fortune, 1989).

More recently, the effect that the dominant follicle has on ovarian follicular dynamics during naturally occurring estrous cycles of dairy heifers has been reported (Ko *et al.*, 1991). These investigators observed that the onset of regression of the largest subordinate follicle of the first wave was delayed by cauterization of the concomitant dominant follicle on day 3 of the cycle. Further, emergence of the second wave was hastened when the dominant follicle of the first wave was cauterized on either days 3 or 5 of the cycle. Collectively, these experimental data support the premise that a dominant follicle causes regression of subordinate follicles and that during its growing phase, a dominant follicle suppresses the emergence of a succeeding wave of follicles.

The mechanism(s) employed by the dominant follicle to suppress growth of subordinate follicles is not clear, however, follicular fluid contains numerous substances that may act as intragonadal regulators of folliculogenesis. In addition to ovarian steroids, follicular fluid from various species has been found to contain inhibin, activin, follistatin, glycosaminoglycans, oxytocin, prorenin, renin, angiotensin, substance P, luteinization inhibitor, gonadotropin binding inhibitors, placental proteins, growth factors, plasminogen activator, and oocyte maturation inhibitor (for review, see Tonetta and diZerega, 1989). Of those factors listed, inhibin has probably received the most attention because it is a strong

negative regulator of FSH secretion and may have paracrine functions in the regulation of preovulatory follicular development (Ying, 1988; Hillier, 1991). Structurally, bovine inhibin is a glycoprotein heterodimer of two subunits ($\alpha$ and $\beta$) and occurs in two forms ($\alpha$CA and $\alpha$-43) which share the same $\beta$-subunit but $\alpha$-subunits of different lengths (for review see, Findlay *et al.*, 1991). Inhibin was first detected in bovine FF by DeJong and Sharp (1976) and acts to preferentially inhibit the synthesis and/or secretion of FSH from the anterior pituitary (Burger, 1988). More specifically, *in vivo* administration of bovine FF (containing inhibin activity) suppressed production of messenger RNA (mRNA) for the $\beta$-subunit of FSH in the pituitary and decreased circulating concentrations of FSH in cattle (Beard *et al.*, 1989). Because follicular recruitment is a gonadotropin-dependent event (Goodman and Hodgen, 1983) and FSH receptors are present in both theca and granulosa cells of preantral follicles (Richards, 1980), it has been suggested that inhibin produced by large dominant follicles may affect the growth and development of smaller subordinate follicles by depriving them of the FSH stimulus required for further development, resulting in atresia. Further, concentrations of LH receptors from granulosa cells (Walters *et al.*, 1982b; Ireland and Roche, 1983b) and theca cells (Ireland and Roche, 1983b) of large antral follicles increase prior to the preovulatory surge of LH and decline shortly before ovulation in cattle. Collectively, these data suggest that large preovulatory follicles aid in the demise of subordinate follicles, and because these dominant follicles are more sensitive to LH (increased receptor number) they are induced to ovulate by the preovulatory surge of this gonadotropin.

In addition to the endocrine function of inhibin described above, Hillier (1991) has proposed a model in which granulosa-derived inhibin of the preovulatory dominant follicle

acts in a paracrine fashion on its own theca interna to increase androgen synthesis. Granulosa cells aromatize the androgen into estradiol which is secreted into the peripheral circulation and acts at the pituitary to reduce FSH secretion. Therefore, according to this model, inhibin-induced estrogen synthesis as well as inhibin itself act indirectly to suppress the development of subordinate follicles. Currently, inhibin-related research is aimed at determining the exact role(s) this glycoprotein plays in folliculogenesis, as well as, developing practical applications of this research that would provide physicians and livestock producers with new methods for regulating ovulation in women and domestic species, respectively.

***Follicular Atresia***

Follicular atresia is the process whereby follicles degenerate and are eliminated from the pool of nongrowing and growing follicles available for recruitment and selection, respectively. The percentage of follicles undergoing atresia has been summarized by species (Byskov, 1978). Greater than 99 percent of ovarian follicles of humans, monkeys, dogs and guinea pigs have been estimated to undergo atresia. This percentage is lower in the case of mice (77%) and rats (75%) but still demonstrates that the majority of, and in some cases nearly all, ovarian follicles undergo atresia.

Canine follicles have been reported to undergo two types of atresia (Spanel-Borowski, 1981). Pre-antral follicles exhibited **type A atresia** in which necrotic changes occurred predominantly in the oocyte and zona pellucida. Antral follicles, on the other hand, exhibited **type B atresia**, characterized by profound degenerative changes in granulosa cells with little or no effect on the oocyte or zona pellucida. It appears that in the immature hypophysectomized estrogen-primed rat, at least, LH-induced androgen from the theca

interna may bind to granulosa cell androgen receptors to induce atresia in pre-antral follicles (Schreiber *et al.*, 1976), however, the exact mechanism that leads to cell death is unknown. Theca-derived androgen may mediate atresia in antral (Graafian) follicles as well because immunization of ewes against androstenedione increased ovulation rate (Scaramuzzi *et al.*, 1977). Although the mechanism responsible for the increase in ovulation rate was not determined, the authors speculated that neutralization of androstenedione activity may reduce estrogen synthesis by granulosa cells or prevent an inhibitory action of androstenedione on the hypothalamic-pituitary axis. In either case, active immunization against the androgen increased systemic levels of LH during the estrous cycle which presumably resulted in the presence of more non-atretic antral follicles at the time of ovulation as compared to control ewes. Terranova (1981) proposed that antral follicles become atretic when theca interna cells shift from synthesizing androgen to progesterone, thereby depleting the granulosa cell of estrogen precursor, leading to granulosa cell and oocyte degeneration. There is evidence to suggest that lack of estrogen rather than increase in androgen is the principle factor responsible for atresia in preovulatory follicles in the rat (Dhanasekaran and Moudgal, 1989). These investigators speculate that decreased follicular estrogen results in the activation of lysosomal degradation of granulosa cells and that estrogen may regulate synthesis of lysosomal enzymes in this tissue.

More recently, it has been suggested that apoptosis, programmed cell death, may be the mechanism underlying follicular atresia (for review, see Hurwitz and Adashi, 1992). Apoptosis is characterized by the internucleosomal cleavage of genomic DNA, by a $Ca^{2+}/Mg^{2+}$-dependent endogenous endonuclease, into fragments that form a distinctive ladder pattern after electrophoretic separation. Internucleosomal cleavage of DNA was

observed in atretic, but not normal, follicles from chickens and pigs (Tilly *et al.*, 1991) and rats (Hughes and Gorospe, 1991). Further, apoptosis may induce the demise of theca as well as granulosa cells in atretic chicken follicles because both cell types exhibited DNA fragments characteristic of the process (Hurwitz and Adashi, 1992). Collectively, these data support the concept that apoptosis is involved in the induction of follicular atresia; however, to date, the signal responsible for activating the apoptotic process, thus inducing follicular atresia, is not known.

Follicular atresia is characterized morphologically by the appearance of pyknotic nuclei in granulosa cells and subsequent degeneration of the membrana granulosa (for review see: Greenwald and Terranova, 1988). Atresia in the oocyte is characterized in many species by breakdown of the germinal vesicle, chromosome alignment in metaphase and on occasion, expulsion of a polar body. Theca cells also show signs of atresia but these differ greatly between species. For example, the theca layer of atretic follicles of cows and sheep regresses completely, in contrast to rodents and primates in which the theca undergoes hypertrophy. Atretic follicles of cows can be identified by gross visual inspection of the theca interna. Pink to dark red theca is associated with healthy non-atretic follicles whereas a colorless theca interna indicates an advanced stage of follicular degeneration (McNatty *et al.*, 1985).

Biochemical markers of atresia have been identified in the follicular fluid of most species. In the cow, increased ratio of progesterone to estrogen in follicular fluid (McNatty *et al.*, 1984a; Ireland and Roche, 1983a,b) and decreased capacity of theca interna to respond to LH with synthesis of cAMP and androgen ( McNatty *et al.*, 1985) is associated with morphological signs of atresia. In addition, increased concentrations of the

glycosaminoglycans chondroitin sulfate (Bellin and Ax, 1984) and heparan sulfate (Bushmeyer *et al.*, 1985) in bovine follicular fluid have also been identified as markers of atresia. More recently, both renin and prorenin (regulators of cardiac homeostasis) have been identified in bovine follicular fluid and increased levels of prorenin were reported to be a valid indicator of follicular atresia in this species (Mukhopadhyay *et al.*, 1991). Although a number of compounds found in follicular fluid have been associated with atresia, it is not yet known if these compounds play a role in the induction of atresia or are merely present as by-products of this process.

**Ovulation**

Ovulation is the process by which the ovum is released from the nurturing environment of the follicle. Structural and biochemical characteristics as well as hormonal regulation of this ovarian event have been reviewed (Espey, 1978; Peters and McNatty, 1980; Lipner, 1988). An overwhelming amount of the research conducted in this area has used various laboratory animals as the experimental model of choice, therefore, the literature cited in the following paragraphs is predominantly concerned with ovulation in rats, mice and rabbits. However, pertinent literature pertaining to cattle, or other domestic ruminants, is included when available. Briefly, the dominant Graafian follicle is stimulated to rupture by the preovulatory surge of LH. As ovulation approaches, the follicle becomes more vascular and protrudes further from the surface of the ovary until, finally, the apical portion of the follicle, often marked by a stigma, ruptures releasing the oocyte. Although the process of ovulation can be described in very simple terms, there are many complex structural and biochemical changes that occur in the follicle prior to ovulation (Espey, 1978).

In addition, neuroendocrine regulation of the preovulatory surge of LH, in mammals, is complex and appears to be regulated by the interaction of neuropeptide Y (NPY), catecholamines, endogenous opioid peptides (EOP) and gonadal steroids in the brain stem and hypothalamus (Kalra and Crowley, 1992).

***Structural and Biochemical Changes***

In order for the oocyte to leave the follicle at ovulation the integrity of the follicle wall must be destroyed. The apex of the follicle (rupture site) is comprised of several layers of tissue that must be degraded in order for the oocyte to escape and includes the outermost layer of surface epithelium and its associated collagenous basement membrane, a meshwork of tunica albuginea and theca externa layers that consists of fibroblasts and collagen fibers and, finally, the basal lamina of the granulosa cell layer. The follicular wall is thinnest at the apex and, presumably, that is why rupture occurs at this site (Espey, 1978). The theca interna layer of the follicle is highly vascularized and the follicle receives the greatest volume of blood just prior to ovulation. Enhanced clearance of fluid from highly permeable capillaries (Moor *et al.*, 1975) in conjunction with changes in the collagen-fibroblast matrix (Espey, 1978) results in the marked increase in follicular fluid volume and follicular size observed near the time of ovulation.

The surge of LH preceding ovulation is responsible for initiating a number of biochemical changes in the follicle that result in degradation of the follicle wall and release of the oocyte. Luteinizing hormone appears to exert its effects on the preovulatory follicle by initiating the synthesis of steroid and prostaglandins as well as specific proteins involved in collagenolysis (Lipner, 1988). Effects of LH at ovulation are probably mediated through the action of cAMP because administration of forskolin (a receptor-independent adenylate

cyclase activator) to perfused rabbit (Holmes *et al.*, 1986) or pregnant mare serum gonadotropin (PMSG)-primed rat (Brännström *et al.*, 1987) ovaries stimulates ovulation in both species. Intrafollicular injections of protein synthesis inhibitors (actinomycin D and cycloheximide) have been reported to inhibit ovulation in the rabbit (an induced ovulator) when administered within 5 hr of mating, as well as, the hamster (Lipner, 1988). These data suggest that follicular protein synthesis is a necessary requirement for ovulation and is presumably mediated by rising levels of LH as time of ovulation approaches.

The role of ovarian steroids in ovulation has been investigated using specific inhibitors of aromatase (4-hydroxy-androstenedione) and 3$\beta$-HSD [17$\alpha$-(3'-hydroxypropyl)-1,3,5,6,8(9)-oestropentaene-3,17$\beta$-diol; Compound A] enzymes. Administration of the aromatase inhibitor to perfused rat ovaries inhibited LH-induced estrogen production but did not inhibit ovulation (LeMaire, 1984). Similar studies using the 3$\beta$-HSD inhibitor, Compound A, in perfused immature rat ovaries, resulted in an inhibition of ovulation that could be reversed by progesterone but not testosterone (Brännström and Janson, 1989). In the ewe, intrafollicular injection of a 3$\beta$-HSD inhibitor, isoxazol, inhibited ovulation and the effect could be reversed by administration of progesterone or $PGF_{2\alpha}$ but not $PGE_2$ (Murdoch *et al.*, 1986). Additional support for the involvement of progesterone in ovulation comes from experiments in which ovulation was blocked in PMSG-primed immature rats immunized against progesterone (Mori *et al.*, 1977). This effect could also be reversed by subsequent administration of progesterone. Collectively, these data support the concept that progesterone synthesis may be prerequisite for ovulation in rodents and sheep.

Prostaglandins of the E and F series (products of the cyclooxygenase pathway) are synthesized locally in the ovary of most mammals, and appear to be regulated by

gonadotropins. Reversible inhibition of ovulation in rabbits and rats using cyclooxygenase inhibitors (indomethacin or aspirin) has been reported (LeMaire, 1989). Brännström and coworkers (1987) demonstrated that indomethacin inhibited forskolin-induced ovulation in PMSG-primed perfused rat ovaries and that the inhibition could be reversed by addition of prostaglandin $E_2$ ($PGE_2$; 1 $\mu$g/ml) to the perfusion medium. In addition, indomethacin has been shown to inhibit gonadotropin-releasing hormone (GnRH)-induced ovulation in hypophysectomized rats (Eckholm *et al.*, 1982). In sheep, injection of indomethacin (100 $\mu$g) directly into the preovulatory follicle effectively inhibited ovulation and, as was the case in the rat, the inhibition could be reversed by systemic injection of $PGE_2$ or $PGF_{2\alpha}$ (Murdoch *et al.*, 1986). Collectively, these data support the supposition that prostaglandins are involved in the ovulatory process in rodents and sheep, however, they do not indicate if these eicosanoids are essential for ovulation.

Presently, the exact function of prostaglandins in the induction of ovulation is not well understood, however, it has been proposed that prostaglandins are involved in the activation of proteinases responsible for structural degradation of the follicle wall (Espey *et al.*, 1981). Inhibition of prostaglandin synthesis with indomethacin has been shown to attenuate follicular collagenolysis in rodents (LeMaire, 1989) and sheep (Murdoch *et al.*, 1986; Murdoch and McCormick, 1991). Effects of indomethacin on follicular prostaglandin synthesis, steroidogenesis, collagenolysis and ovulation in sheep were recently reported (Murdoch and McCormick, 1991). These investigators determined that systemic administration of indomethacin (500 mg), to synchronized ewes induced to ovulate with a GnRH agonist, inhibited ovulation and the LH-induced rise in follicular $PGF_{2\alpha}$ but did not alter systemic preovulatory patterns or follicular tissue concentrations of estradiol-17$\beta$,

testosterone or progesterone. A lower dose of indomethacin (100 mg), administered to similarly treated ewes, was also effective in inhibiting the rise of follicular $PGF_{2\alpha}$, however, ovulation as well as the secretory patterns and concentrations of steroids measured, were unaffected. Further, follicular collagenase activity was decreased in ewes that received 500 mg indomethacin as compared with control ewes and ewes receiving the 100 mg dose. It should be noted that the essentiality of prostaglandins in the ovulatory process of sheep could not be established in this experiment because intrafollicular prostaglandin synthesis was not completely inhibited by indomethacin (follicular $PGF_{2\alpha}$ was still detectable after indomethacin treatment). Data concerning the higher, but not lower, dose of indomethacin are in agreement with an earlier study in which intrafollicular administration of indomethacin (100 $\mu$g) inhibited ovulation and reduced follicular collagenase activity in ewes. Blockade of ovulation could be overcome and collagenase activity restored by systemic administration of $PGE_2$, $PGF_{2\alpha}$, or intrafollicular injection of bacterial collagenase (Murdoch *et al.*, 1986). Collectively, these data demonstrate that high levels of indomethacin reduce follicular collagen breakdown and inhibit ovulation in ewes. However, Murdoch and McCormick (1991) suggested that indomethacin-induced inhibition of ovulation in sheep may not be primarily due to inhibition of LH-induced biosynthesis of follicular prostaglandins because, although both high and low doses of indomethacin attenuated the preovulatory rise in $PGF_{2\alpha}$, ovulation and collagenolysis was impaired only in those ewes receiving 500 mg of the nonsteroidal antiinflammatory drug.

Enzymatic degradation of the follicular wall is generally accepted to be the means of follicular rupture at ovulation and follicular prostaglandin, in conjunction with LH and FSH, may assist in the degradation of follicular wall collagen by invoking the production

of a specific protease, plasminogen activator, that initiates a cascade of enzymatic events within the ovulatory follicle. The **plasminogen-activator, plasminogen hypothesis** has been proposed to explain the mechanism underlying follicle rupture (for references, see Lipner, 1988). Plasminogen activator (PA) is present in the follicular fluid and follicular wall extracts of many species, including the bovine, and is thought to be synthesized by granulosa cells because granulosa cells cultured with FSH or LH *in vitro* produced the enzyme. Two different types of plasminogen activator are known to exist in mammals, and granulosa cells produce both types, urokinase PA (uPA) and tissue PA (tPA), however, follicles contain greater concentrations of uPA and it is thought that this enzyme is responsible for plasminogen breakdown in the follicle. Cyclic AMP and $PGE_1$ and $PGE_2$, which act via cAMP, provoke follicular biosynthesis of PA and therefore this cyclic nucleotide is thought to mediate the prostaglandin-induced synthesis of PA that is observed prior to ovulation. Plasminogen, also present in follicular fluid, is a serine protease produced by the liver and occurs in the blood in high concentrations. Plasminogen is substrate for both types of PA and is thought to enter the follicular fluid from the vasculature of the theca layer. Plasminogen is cleaved by PA and generates another protease, plasmin. Plasmin in turn cleaves latent (inactive) collagenase, presumably synthesized by granulosa cells, to form active collagenase. Active collagenase degrades collagen into telopeptide-free collagen, which is further degraded by nonspecific proteases until the follicle ruptures. In addition, it has been hypothesized that intrafollicular $PGF_{2\alpha}$ may contract follicular wall smooth muscle fibers localized in the theca externa, to aid in expulsion of the oocyte.

*Oocyte Maturation*

Prior to expulsion from the follicle, the preovulatory surge of LH induces germinal vesicle breakdown (GVBD) and resumption of meiosis in the oocyte. Oocytes are maintained in the dictyate stage from fetal life until ovulation, presumably, through the action of a factor, of granulosa or cumulus cell origin, that inhibits maturation: **oocyte maturation inhibitor** (OMI; for review, see Tonneta and diZerega, 1989). The mechanism behind meiotic arrest is not well understood, but, results from *in vitro* experiments have implicated cAMP in the activation or production of the putative inhibitor in mouse oocytes (Eppig *et al.*, 1983) because continuous exposure to cAMP or cAMP derivatives (Cho *et al.*, 1974) prevents spontaneous maturation (denoted by GVBD) when oocytes are removed from their intrafollicular environment. Further, exposure of mouse oocyte-granulosa cell complexes to a membrane-permeable cAMP antagonist Rp-cAMPS, (Rp-adenosine-3′-5′ cyclic phosphorothioate) that competes with cAMP for the regulatory subunit of protein kinase A, induced oocyte maturation (Eppig, 1991). More recently, participation of a purine metabolic pathway enzyme, inosine monophosphate (IMP) dehydrogenase, (IMP → xanthosine monophosphate) was shown to be essential for the maintenance of meiotic arrest in murine oocytes both *in vivo* (Downs and Eppig, 1987) and *in vitro* (Eppig, 1991) suggesting that guanyl or xanthyl derivatives play a role in maintaining oocyte meiotic arrest.

As was observed with rodent oocytes, maturation was inhibited in bovine oocytes treated with cAMP *in vitro* (Sirard and First, 1988). In addition, when oocytes were incubated *in vitro* with hypoxanthine or adenosine the majority of oocytes underwent GVBD and resumed meiosis, contrary to data that was reported for mouse oocytes treated with

hypoxanthine (Eppig, 1989) and suggests that the role of purines or regulation of purine biosynthetic pathways during oocyte maturation may vary between species.

Protein synthesis has been reported to be required for GVBD in bovine oocytes *in vitro* (Hunter and Moor, 1987) and may also be required for progression into metaphase I of meiosis because the formation of a normal meiotic spindle was prevented when oocytes were treated with cycloheximide (protein synthesis inhibitor) after GVBD had already occurred (Sirard *et al.*, 1989). In addition, a more recent *in vitro* study investigated the changes in protein synthesis and phosphorylation patterns during bovine oocyte maturation (Kastrop *et al.*, 1990). Cumulus-oocyte complexes were cultured various lengths of time with [$^{35}$S]methionine or [$^{32}$P]orthophosphate to generate sequential protein synthesis and phosphorylation patterns, respectively. These researchers observed that changes in protein synthesis occurred exclusively after GVBD and that changes in protein phosphorylation patterns occurred predominantly before GVBD, suggesting that protein synthesis is not required for GVBD but may be required for maturational events occurring after GVBD.

The apparent contradiction as to the necessity of protein synthesis for GVBD in bovine oocytes, reported by Hunter and Moore (1987) and Kastrop and coworkers (1990) was resolved when an experiment was conducted to further examine protein synthesis and phosphorylation in bovine oocytes during *in vitro* maturation (Kastrop *et al.*, 1991). Cumulus-oocyte complexes were incubated at various times during the culture period with either an inhibitor of mRNA ($\alpha$-amanitin) or protein (cycloheximide) synthesis. Addition of $\alpha$-amanitin during the first 2 hr of culture prevented the phosphorylation of specific proteins prior to GVBD and decreased the occurrence of GVBD (27%) as compared to control oocytes (97%). Addition of cycloheximide also blocked the phosphorylation of

specific proteins prior to GVBD and reduced the occurrence of GVBD (5%) in nearly all oocytes treated. Although no changes were observed in the pattern of proteins synthesized before GVBD, the authors explain that the observed effect of cycloheximide on protein phosphorylation clearly demonstrates that protein synthesis is necessary for the protein phosphorylation observed prior to GVBD and because this *de novo* synthesis probably represented only a small amount of the total protein synthesized, it may not have been detected in the previous experiment (Kastrop, 1990). Collectively, these data suggest that transcription of mRNA and *de novo* protein synthesis prior to GVBD, are required for complete maturation of bovine oocytes *in vitro*.

It is clear that the mechanism of action of OMI and the interrelationship of this putative inhibitor with cAMP and purine pathway intermediates in oocyte maturation have yet to be determined. Based on the available evidence, it seems likely that OMI may exert its effects through a combination of factors that act in concert to induce meiotic arrest in mammalian oocytes. Further investigation is required if we are to completely understand this complex biological phenomenon.

***Regulation of the Preovulatory Surge of LH***

Regulation of the preovulatory surge of LH is a complex process that requires neuromodulation of GnRH, also referred to as LHRH/FSHRH (luteinizing hormone releasing hormone/follicle stimulating hormone releasing hormone), secretion by catecholamines, steroids and neuropeptides. Neuromodulation of GnRH secretion has been most extensively studied in the rat and monkey although in more recent years interest and methodology has developed to support investigation and quantification of GnRH secretion in large domestic species such as sheep and therefore our understanding of the regulation of gonadotropin

secretion in this species has increased considerably. Regulation of gonadotropin secretion by steroid hormones in various species, including basal episodic LH secretion observed during most of the estrous cycle and the high frequency, low amplitude pulses that constitute the preovulatory LH surge, has been exceptionally well reviewed (Brann and Mahesh, 1991a). Control of preovulatory LH release and the growing role of neuropeptide Y in the regulation of this process has also been reviewed in exceptional detail in two recent publications (Kalra, 1986; Kalra and Crowley, 1992).

Gonadotropin-releasing hormone, as the name suggests, stimulates the release of LH, and to a lesser extent FSH, from gonadotroph cells localized in the adenohypophysis (anterior pituitary). The distribution of GnRH neurons in the central nervous system (CNS) appears to differ between species and has been reviewed (Silverman, 1988). The most prominent network of GnRH cell bodies forms, in most species, a continuum from the telencephalic diagonal band of Broca, dorsal septal nuclei (medial and triangular septal nuclei) and diencephalonic areas (periventricular, medial and lateral preoptic areas). Clusters of GnRH cells also reside in the lateral hypothalamus and suprachiasmatic nucleus. In the bovine (Dees and McArthur, 1981), and other species (guinea pig, rabbit, monkey, and human) GnRH neurons are found throughout the medial basal hypothalamus (MBH; arcuate nucleus, dorsal median eminence, and premamillary nuclei) in addition to those described in the telencephalon/diencephalon areas, however, they represent only a small proportion of total GnRH cells. Evidence as to the presence or absence of GnRH neurons in the MBH (arcuate nucleus) of the rat is controversial (Silverman, 1988). Similarly, the presence of GnRH perikarya in bovine MBH has been disputed (Leshin *et al.*, 1988). It is agreed, however, that the network of GnRH perikarya that are most directly involved with

LH secretion terminate on fenestrated capillaries in the primary portal plexus of the median eminence (ME) and release GnRH into the portal blood supply feeding the anterior pituitary. Two major GnRH tracts innervate the median eminence in bovine brain (Leshin *et al.*, 1988).

Using vital dyes, Wysloki and King (1936) described the nature of blood flow from the ME to the pituitary in monkeys, cats and rabbits. Briefly, they found that blood entered the primary portal plexus in the ME from superior hypophysial arteries and subsequently drained through long portal vessels to a secondary capillary plexus in the pars distalis (anterior pituitary), clearly establishing a vascular route through which hypothalamic releasing factors could access target pituitary cells directly.

Gonadotropin releasing hormone is a decapeptide (pyro-Glu$^{1}$-His$^{2}$-Trp$^{3}$-Ser$^{4}$-Tyr$^{5}$-Gly$^{6}$-Leu$^{7}$-Arg$^{8}$-Pro$^{9}$-Gly$^{10}$-$NH_2$; Amoss *et al.*, 1971; Matsuo *et al.*, 1971), synthesized in cell bodies of GnRH neurons as a preprohormone (Seeberg and Adelman, 1984) and is cleaved to its bioactive form prior to secretion from the nerve terminal. Release of GnRH is pulsatile and has been shown to evoke pulsatile release of LH from pituitary gonadotrophs in all mammals examined to date. The neuronal system(s) that is responsible for rhythmic activation and subsequent release of GnRH from neurosecretory cells is commonly referred to as the **GnRH pulse generator**, and although the cellular nature of this putative generator has yet to be identified, major inroads have been made in identifying neuroregulatory factors that influence its activity (Knobil, 1990).

Gonadotropin secretion is regulated in part through the combined actions of estrogen and progesterone at the hypothalamus (modulation of GnRH pulse generator) and pituitary (direct effect on gonadotrophs) and these steroids may be either stimulatory or inhibitory in

nature depending on stage of the estrous cycle and associated systemic concentration of gonadal steroids. Estrogen, of follicular origin, is considered to be the primary stimulus for the preovulatory LH surge but in recent years it has become clear that progesterone action is critical for regulation of the magnitude and duration of the surge in some species (Kalra, 1986; Brann and Mahesh, 1991a). Early experiments clearly demonstrated the negative feedback effects of ovarian steroids on gonadotropin secretion because ovariectomy of monkeys (Dierschke *et al.*, 1970), rats (Gay and Sheth, 1972), cows (Hobson and Hansel, 1972), sheep (Clarke and Cummins, 1982) and other species (for review, see Fink, 1988) resulted in elevated concentrations of systemic LH that were pulsatile in nature. Frequency of LH pulses following ovariectomy varied among species, for example, sheep (Butler *et al.*, 1972), cows (Forrest *et al.*, 1980) and monkeys (Dierschke, 1970) exhibited about 1 pulse per hour whereas rats exhibited about 1.5 pulses per hour (Levine *et al.*, 1991). Further, the post-ovariectomy rise in LH secretion observed in the monkey, rat, cow and sheep could be attenuated by subsequent administration of estradiol, thereby providing further evidence of estrogen negative feedback in these species.

In some species, the negative feedback effect of estradiol appears to be mediated at both the hypothalamic and pituitary level. In rats, estradiol has been shown to inhibit GnRH secretion from the hypothalamus *in vivo* and LH secretion from pituitary cells *in vitro*. For example, administration of estradiol to ovariectomized (OVX) rats attenuated the expected increase in proGnRH mRNA in the brain (Toranzo *et al.*, 1989) and estradiol suppressed gonadotropin release in rat pituitary cell cultures treated with GnRH (Moll and Rosenfield, 1984) suggesting that estradiol acts at these two sites in the brain, in this species, to regulate LH secretion. In OVX ewes and heifers, the negative and positive feedback effects of

estradiol result in a biphasic release of LH that is characterized by a transient suppression (negative feedback) and subsequent surge (positive feedback) of this gonadotropin after administration of estradiol. Secretion of LH, in OVX ewes treated with estradiol (50 $\mu$g, i.m.), decreased significantly within 2 hr of injection and remained suppressed for approximately 8 to 10 hr and then increased prior to the LH surge which occurred about 12 to 20 hr after injection (Coppings and Malven, 1976). In contrast, serum LH concentrations in OVX heifers were suppressed between 2 and 6 hr after administration of estradiol (as a silastic implant) and reached maximum concentrations (surge) at 18.5 hr after implant insertion (Beck and Convey, 1977).

Clarke and coworkers (1982) have defined three types of feedback effects that occur after estrogen treatment in OVX sheep. **Short-term negative feedback** is characterized by the immediate reduction of systemic LH concentration after estradiol injection. A **positive feedback** mechanism is then invoked and LH levels rise considerably above preinjection values. Finally, with continued estradiol treatment, **long-term (tonic) negative feedback** maintains LH levels below that of untreated OVX animals. Further, Clarke suggested that these classifications may be applied to normal cyclic events in the ewe. Short-term negative feedback effects of estradiol might function during the late follicular phase of the estrous cycle. Positive feedback effects of estradiol appear to be responsible for initiation of the preovulatory surge of LH and long-term negative feedback produces patterns of gonadotropin secretion similar to that observed during the luteal phase of the cycle and seasonal anestrous. Short-term negative and positive feedback effects of estradiol and their pertinence to the ovulatory process will be discussed in the following paragraphs, whereas long-term negative

feedback will be addressed later in relation to the regulation of LH during the luteal phase of the cycle.

Although early studies clearly demonstrated a biphasic response to estradiol treatment in cows and sheep, the site(s) of estrogen action could not be determined. More recently, the site of estrogen action in short-term negative feedback of LH secretion in sheep and cattle has been further elucidated. In cattle, it has been proposed that estradiol acts initially at the pituitary to suppress LH secretion and later at the hypothalamus or an extra-hypothalamic site to maintain this suppression because pituitary response of OVX heifers to a GnRH challenge (40 $\mu$g) was significantly lower, as compared with control heifers, at 2.5 hr but not 5 hr after estradiol injection (1 $\mu$g/kg body weight) although serum LH was still suppressed at this latter time period (Hinshelwood *et al.*, 1986). These data provide evidence that pituitary responsiveness to GnRH, in the cow, is affected by estradiol treatment and suggest that suppression of LH secretion in the face of restored pituitary responsiveness may result from estradiol-induced alteration of GnRH secretion from the hypothalamus. It should be noted, that unlike the rat, ewe, and monkey, hypophysial portal blood has never been sampled in cows and therefore the effect of ovarian steroids on GnRH secretion from the hypothalamus of OVX or intact animals has not been determined directly. However, because the estrous cycles of the cow and ewe are similar in nature, it seems likely that regulation of gonadotropin secretion by ovarian steroids may be comparable in these two species.

Development of techniques for the serial sampling of hypophysial portal blood in rats (Levine and Ramirez, 1980), sheep (Clarke and Cummins, 1982; Levine *et al.*, 1982; Caraty and Locatelli, 1988), and monkeys (Levine *et al.*, 1985) has allowed concomitant

measurement of GnRH and LH secretion under a variety of experimental conditions and has advanced our understanding of steroid regulation of gonadotropin secretion in these species. In sheep, several studies have suggested that the short-term negative feedback action of estradiol is exerted directly at the pituitary to suppress LH secretion. In one experiment, OVX ewes were injected with estradiol benzoate (50 $\mu$g, i.m.) and portal as well as jugular blood samples were collected during both the negative and positive feedback phases for determination of GnRH and LH, respectively (Clarke and Cummins, 1985). These researchers reported that pulsatile GnRH secretion continued during the negative feedback phase of estrogen treatment (suppressed LH secretion) and was similar to that of untreated control ewes. Further, during the positive feedback phase (LH surge), GnRH pulse frequency was increased and average concentrations of GnRH in portal blood were higher than those in untreated control ewes. In a similar experiment, Schillo *et al.* (1985) also detected GnRH pulses in hypophysial portal blood collected during the period of reduced LH secretion in OVX ewes injected with estradiol benzoate (50 $\mu$g, i.m.). Further evidence supporting the hypothesis that short-term negative feedback of estradiol is mediated at the level of the pituitary in the ewe, was provided by Clark and Cummins (1984) using OVX ewes in which the pituitary had been surgically disconnected from the hypothalamus (hypothalamo-pituitary disconnection, HPD) to eliminate potential hypothalamic effects of steroids on GnRH secretion. After HPD, gonadotropin secretion was restored, to that normally observed in OVX ewes, by the infusion of hourly pulses of GnRH (500 ng) through intra-atrial cannulae. Injection of estradiol benzoate (50 $\mu$g, i.m.) resulted in the suppression of LH between 4 and 8 hr and surge of LH 19 to 28 hr after estrogen treatment and the biphasic release pattern was similar to that observed in estrogen-treated OVX ewes,

except that the peak height of the LH surge was somewhat lower. In contrast, Coppings and Malven (1976) reported that only the initial 4 hr of reduced LH secretion observed after estradiol injection (50 $\mu$g) of OVX ewes was due to a reduction in pituitary sensitivity to GnRH and that the latter 4 to 6 hr of inhibition was due to reduced hypothalamic secretion of GnRH. The authors reached this conclusion because pituitary responsiveness to repeated GnRH challenge (300 ng, i.v. every 2 hr) following estradiol treatment was attenuated for only the first 4 hr of LH suppression and was similar to that observed in control ewes by 6 hr after estradiol treatment even though LH remained suppressed for 8 to 10 hr. Interestingly, results of a recent study (Caraty *et al.*, 1989), in which portal GnRH and peripheral LH secretion were monitored concomitantly in OVX ewes treated with estradiol (50 $\mu$g total: 25 $\mu$g i.v. and 25 $\mu$g i.m.), support the hypothesis of Coppings and Malven (1976) in which estradiol was proposed to act initially at the pituitary and then at the hypothalamus to reduce LH secretion. During the negative feedback phase GnRH pulses were observed even though LH pulses were attenuated by estrogen treatment, suggesting that estradiol was acting on the pituitary to reduce responsiveness to GnRH. However, both frequency and amplitude of GnRH pulses as well as rate of GnRH secretion were lower during the negative feedback as compared with positive feedback phase and this suggested that estradiol was also acting at the hypothalamus to suppress GnRH secretion during the negative feedback phase. The disparity in results obtained in this study and that of Clarke and Cummins (1985) may have resulted from differences in the administration of estradiol (i.v. + i.m. versus i.m only) because LH appeared to be more rapidly attenuated after the i.v. and i.m. injections (Caraty *et al.*, 1989) as compared with the i.m. injection alone (Clarke and Cummins, 1985). It seems reasonable to expect that a greater concentration of

estradiol would reach the hypothalamus and pituitary more rapidly following i.v. injection as compared with the slower diffusion rate from muscle tissue, however, neither study reported the average time from estradiol injection to the observed suppression of LH secretion. Collectively, these studies provide convincing evidence that, in the ewe and the cow, the primary action of estrogen during short-term negative feedback is to reduce pituitary responsiveness to GnRH, but following the initial reduction in gonadotropin secretion, estradiol may also act at the hypothalamus to reduce GnRH secretion and thereby maintain LH suppression.

It is now generally accepted that estrogen and progesterone mediate their effects in mammalian target tissues by binding with high affinity to specific receptors localized in the nucleus of the cell (King and Greene, 1984; Perrot-Applanat *et al.*, 1985). Upon binding hormone the receptor becomes activated, through a conformational change, and is then able to bind with high affinity to nuclear acceptor sites on the chromatin. In this manner steroids regulate gene expression and protein synthesis at the level of transcription or post-transcriptional events (for review, see Yamamoto, 1985; Rories and Spelsberg, 1989). Initial research suggested that free steroid receptors were primarily located in the cytosol and upon binding ligand the steroid-receptor complex migrated to the nucleus (Gorski *et al.*, 1968). It now appears that the presence of cytosolic receptors was an artifact of the tissue homogenization and centrifugation processes, but, because determinations of both cytosolic and nuclear receptors were reported in earlier literature they will be similarly reported here when necessary.

Because steroids mediate their actions through specific receptors and changes in concentration of the number of receptors present in target cells may indicate changes in the

sensitivity of the cell to a particular hormone, it was of interest to determine the concentration of pituitary estrogen receptors associated with LH and FSH response to estradiol injection in OVX ewes (Clarke *et al.*, 1982). In this study, total pituitary estrogen receptor concentration (cytosolic + nuclear) was not significantly altered after estrogen treatment suggesting that the initial reduction in plasma LH and FSH concentrations and subsequent surge of LH are not directly attributable to changes in total estrogen receptor number in this gland. In contrast, estradiol has been demonstrated to increase the concentration of pituitary GnRH receptors in the ewe *in vivo* (Clarke *et al.*, 1988) and in ovine pituitary cells cultured *in vitro* (Gregg *et al.*, 1990). Clarke and coworkers (1988) reported that pituitary GnRH receptor concentrations of OVX-HPD ewes given hourly GnRH pulses (250 ng) increased significantly by 6 hr after an injection of estradiol (50 $\mu$g, i.m.) and were further increased by 16 and 20 hr after treatment. Mean plasma LH concentration and amplitude of LH pulses in response to GnRH were reduced from 4 to 6 hr after estrogen treatment, however, while average plasma LH levels were increased from 14 to 16 and 18 to 20 hr after estrogen, no similar increase in the amplitude of LH pulses was observed during the latter sampling periods. These authors hypothesized that the estradiol-induced increase in pituitary GnRH receptor concentration that occurs during the period of reduced LH secretion may act as a negative feedback "clamp" allowing GnRH to prime pituitary synthesis of LH without provoking its release. In this manner pituitary stores of LH may be increased prior to the LH surge, a phenomenon that has been reported to occur in proestrous (period of the estrous cycle beginning with regression of the CL and extending to the initiation of estrus) sheep (Roche *et al.*, 1970). Similarly, pituitary content of mRNA

for LH-$\alpha$ and -$\beta$ subunits is increased in intact ewes during the preovulatory surge, as compared with other times during the estrous cycle (Landefeld *et al.*, 1985a,b).

Somewhat similar results, with respect to serum and pituitary concentrations of LH, have also been reported for OVX Zebu-cross cows treated with estradiol (Schoeneman *et al.*, 1985). Serum concentrations of LH were suppressed for 3 hr after administration of estradiol (1 mg, i.m.) and then peaked in a preovulatory-like surge about 21 hr after treatment. Pituitary concentrations of LH were significantly increased at 20 hr after treatment, just prior to the observed LH surge, and then declined. Contrary to the results described above for the OVX-HPD ewe (Clarke *et al.*, 1988), concentrations of pituitary receptors for GnRH were not significantly increased early in the negative feedback phase of LH secretion but were maximal at 12 hr after estrogen treatment; several hours in advance of the LH surge. Similar results have been reported in intact ewes prior to the LH surge (Crowder and Nett, 1984) and therefore the observed differences among these experiments may be the result of inappropriately timed sampling or the presence of endogenous GnRH in OVX cows as well as OVX and intact ewes. Clearly, however, concentrations of GnRH receptors do increase prior to the LH surge in OVX and intact ewes and cows and may result in enhanced pituitary sensitivity to the negative as well as positive feedback effects of estradiol at this time. Further, estradiol may enhance the synthesis and accumulation of LH in the pituitary prior to the preovulatory surge.

It is generally accepted, for many species, that the gonadotropin surge observed prior to ovulation is initiated by a positive feedback effect of estradiol at the pituitary to increase its sensitivity to GnRH and perhaps also at the hypothalamus to increase GnRH secretion. Ovariectomy before the day of proestrus in rats, hamsters and sheep, inhibits the

preovulatory surge of gonadotropins and can be reversed by administration of estradiol, thereby demonstrating the importance of this steroid in the regulation of the preovulatory surge of LH and FSH (Brann and Mahesh, 1991a). Early studies in rats (Sarkar *et al.*, 1976) and monkeys (Neill *et al.*, 1977) suggested that GnRH secretion increased at the time of the LH surge. In the ewe, however, when GnRH secretion was monitored throughout the estrous cycle an increase in GnRH secretion prior to the LH surge was not consistently observed in all animals (Clarke *et al.*, 1987) and suggested that an increase in hypothalamic GnRH secretion may not be required to initiate the preovulatory surge. In contrast, more recent experiments suggest that estrogen positive feedback also occurs at the level of the hypothalamus in sheep, because GnRH secretion is enhanced prior to the LH surge in estradiol-treated OVX (Moenter *et al.*, 1990) as well as non-treated cyclic ewes (Moenter *et al.*, 1991). Ewes ovariectomized during the breeding season received progesterone and estrogen implants to simulate the luteal phase of the estrous cycle. Progesterone implants were removed 1 wk later to simulate luteolysis and additional estrogen implants were inserted (to simulate follicular phase levels of estradiol) to induce a predictable surge of LH 20 to 24 hr later. Secretion of GnRH, in response to rising estrogen levels, shifted from regular pulsatile secretion to a surge release that coincided with the surge release of LH, however, it was not possible to determine if the GnRH surge was a result of an increase in pulse frequency, amplitude or a combination of these two factors (Moenter *et al.*, 1990). A subsequent experiment has demonstrated that a massive surge in GnRH (40-fold greater than basal secretion) preceded the LH surge in cyclic ewes and that the pattern of GnRH secretion prior to the surge differed as compared with that observed during the surge. Prior to the surge, GnRH pulses returned to baseline between episodes of release whereas during

the surge GnRH remained continuously elevated with fluctuations imposed on the elevated baseline secretion (Moenter *et al.*, 1991). Dynamics of a GnRH pulse were reported for short-term OVX ewes after the removal of estradiol and progesterone implants, which were used to maintain hourly high amplitude pulses of GnRH and LH similar to luteal phase secretion in intact ewes (Moenter *et al.*, 1992). These researchers determined that the contour of most GnRH pulses approximated that of a square wave. Secretion of GnRH was observed to increase as much as 50-fold within 1 min and the average peak concentration was 70-fold greater than baseline. Further, the release of GnRH was sustained for an average of 5.5 min and decreased to prepulse levels within 3 min. Collectively, these data demonstrate that GnRH secretion in the ewe is dynamic and that altered secretion of this decapeptide from the hypothalamus, in response to estradiol positive feedback, may result in a preovulatory surge of GnRH coincident with that of LH.

Additional support for a hypothalamic action of estradiol in initiating the LH surge has been provided by electrophysiological experiments with ewes (Thiéry and Pelletier, 1981) but not monkeys (Kesner *et al.*, 1987) in which GnRH pulse generator activity was determined by recording the electrical activity of groups of neurons (multi-unit activity; MUA) using tungsten electrodes stereotaxically placed in the anterior median eminence. In the OVX ewe, the firing of GnRH neurons, recorded as bursts of multi-unit activity (MUA), preceded plasma LH pulses and followed a circhoral pattern similar to that of LH release (Thiéry and Pelletier, 1981). After injection of estradiol (100 $\mu$g i.v.) LH secretion was suppressed while MUA was increased demonstrating again that the initial suppression of LH occurs at the level of the pituitary. Results obtained from a second group of OVX ewes that received estradiol (50 $\mu$g i.m.) and were monitored through the surge of LH,

demonstrated a shortening of the MUA circhoral period and a lengthening of bursting activity at the initiation of the LH surge that was followed by an increase in the mean MUA amplitude. These results support the hypothesis of hypothalamic involvement of GnRH neurons in the estradiol-induced LH surge in OVX sheep. In contrast, hypothalamic MUA of estrogen-treated monkeys was reduced several hours after estradiol treatment and did not increase at the time of the LH surge (Kesner *et al.*, 1987). However, differences between species or in neuronal systems recorded may explain the apparent discrepancy in the results of these two studies. Accumulating evidence, for the ewe and cow, suggests that estrogen acts to increase pituitary responsiveness to GnRH and may also increase hypothalamic secretion of GnRH; the combination of these two actions culminating in the surge of LH and ovulation.

The mechanism through which estrogen positive feedback increases pituitary responsiveness to GnRH is thought to result from an increase in the concentration of GnRH receptor prior to the LH surge, as was described previously. However, the positive feedback of estrogen on GnRH secretion does not appear to be mediated through GnRH containing neurons directly because it has been reported in the rat that few GnRH neurons (about 1 of 435) contain estrogen receptors (Shivers *et al.*, 1983). It is therefore more likely that estradiol exerts its affects on GnRH secretion through other neuronal systems that are responsive to estrogen and terminate in close proximity to GnRH releasing neurons. Several neuronal systems that take up estradiol and modulate GnRH secretion have been identified in rat hypothalami and include the steroid concentrating neurons, adrenergic systems, neuropeptide Y (NPY)- and endogenous opioid peptides (EOP)-producing neurons. Presumably, these systems alter the amplitude or frequency of GnRH release through an

alteration in synthesis or secretion of the neurohormones they produce (for references, see Kalra, 1986). In addition, some investigators have suggested that ovarian steroids may interact with specific membrane receptor sites (Pfaff and McEwen, 1983; McEwen *et al.*, 1984) on GnRH neurons to modulate GnRH release directly in a nongenomic manner (Ke and Ramirez, 1987); however, this view has not been widely accepted.

The role of progesterone in the regulation of gonadotropin secretion and ovulation is complex and varies among species. In the cycling rat, progesterone appears to facilitate the preovulatory surge of LH when administered just prior to the surge. However, administration of progesterone earlier in the cycle results in attenuation of the LH surge (Everett, 1948) suggesting that this steroid can be either inhibitory or stimulatory depending on the stage of the cycle. Serum progesterone levels have been demonstrated to rise prior to initiation of the LH surge in rats (Feder *et al.*, 1971), humans (Labode *et al.*, 1976) and monkeys (Schenken *et al.*, 1985) but not in sheep or cattle (Hansel and Echternkamp, 1972; Chenault *et al.*, 1975). Blocking the proestrus rise of progesterone in rats with a 3$\beta$-HSD inhibitor (epostane) suppressed the preovulatory surge of LH and FSH and subsequent injection of progesterone restored the gonadotropin surge (DePaolo, 1988). Progesterone appears to enhance gonadotropin secretion in the rat through a number of different mechanisms. Both *in vivo* and *in vitro* experiments have demonstrated that progesterone increases hypothalamic GnRH content and release, enhances pituitary responsiveness to GnRH and decreases pituitary nuclear estrogen receptor accumulation and therefore antagonizes the short-term inhibitory effect of estrogen on gonadotropin secretion (for references, see Brann and Mahesh, 1991a). Collectively, these data suggest that

progesterone enhances the magnitude of the preovulatory LH surge in the rat and that it does so through several different mechanisms of action.

In the ewe, it has been demonstrated that progesterone inhibits the tonic secretion of LH (Karsch *et al.*, 1977; Hauger *et al.*, 1977). Because the preovulatory rise in estradiol and LH are preceded by declining progesterone secretion from the regressing CL, Karsch and coworkers (1979) conducted an experiment to examine the hypothesis that, in the ewe, progesterone withdrawal releases the hypothalamic-pituitary axis from negative feedback inhibition, thereby permitting a sustained rise in tonic LH secretion, which then stimulates an increase in ovarian estradiol secretion that results in the preovulatory surge of LH. In this experiment, corpora lutea were surgically removed from ewes on day 8 (midluteal phase) of the estrous cycle and 3 silastic packets containing progesterone were implanted (s.c.) to maintain midluteal concentrations of progesterone (3-4 ng/ml) after CL removal. One group of ewes had their CL removed but did not receive progesterone and control ewes underwent surgery but their CL were not removed. In control ewes, the decrease in progesterone observed at luteolysis was associated with a four- to fivefold increase in serum LH and estradiol concentrations followed by the preovulatory surge of LH. Secretion of LH was suppressed in the ewes implanted with progesterone until the implants were removed. Withdrawal of progesterone, by removal of the progesterone implants on day 14 or enucleation of the CL on day 8 without subsequent progesterone treatment, resulted in sustained increases in estradiol and LH that were followed by a preovulatory surge in LH. The time from onset of declining progesterone level to the peak of the LH surge was similar for all groups. These results provide strong evidence to support the hypothesis that

progesterone in this species inhibits tonic LH secretion and that withdrawal of this steroid (luteal regression) permits the necessary increases in estradiol that trigger the surge of LH.

The importance of progesterone during the estrous cycle of the ewe was further investigated in OVX ewes implanted with estradiol and progesterone to produce an "artificial estrous cycle" in which the separate and combined actions of these two steroids could be evaluated (Karsch *et al.*, 1980). Ovaries were removed shortly after ovulation and ewes received one of the following treatments : empty implants, progesterone implants only, estradiol implants only, or estradiol and progesterone implants. Implants were added or removed as necessary to simulate the cyclic patterns and levels of serum progesterone and/or estradiol reported for intact ewes during the cycle. Sequential addition and removal of estradiol and progesterone implants in OVX ewes resulted in circulating levels of steroids that were remarkably similar to those of intact ewes during a cycle. Treatment with progesterone implants alone did simulate the normal pattern of luteal phase progesterone secretion but did not result in luteal phase levels of LH or pattern of LH release that were similar to those observed in intact ewes. Mean levels of LH were greater in the progesterone-treated versus intact ewes but were significantly lower than in untreated OVX control ewes. When progesterone was removed to simulate luteolysis, LH levels increased twofold and resembled the rise in LH that precedes the preovulatory surge of LH in intact ewes. In those animals receiving estrogen implants only, mean level and pattern of serum estradiol did approximate that of intact ewes. However, as was observed with progesterone treatment, the pattern of LH secretion was not similar to that of intact ewes and the mean level of LH was intermediate between those of OVX and intact sheep. Addition of estradiol implants early (days 4 and 5) and late (days 14 and 15) in the absence of progesterone

resulted in small LH peaks on days 5 and 15, respectively. The peak of LH induced by the simulated preovulatory rise in estradiol was smaller than the preovulatory surge of LH in intact ewes and occurred several days earlier. In addition, the estradiol-induced LH peaks were not preceded by the typical sustained increase in basal serum LH observed in intact ewes prior to the LH surge. From these data, these researchers confirmed the hypothesis that both progesterone and estradiol are required to regulate LH secretion during the estrous cycle of the ewe. More specifically, both progesterone and estradiol can suppress tonic LH secretion in the absence of ovaries but neither steroid alone can account for the control of tonic LH secretion during the estrous cycle. Further, the withdrawal of progesterone at luteolysis can account for the sustained increase in LH that precedes the preovulatory surge of LH but the decline in the level of this steroid is not sufficient, in itself, to initiate the preovulatory surge of LH. In addition, the preovulatory rise in estradiol can stimulate the LH surge but, in the absence of progesterone, the surge may not attain its full magnitude. Finally, the rise in basal LH that precedes the surge of LH in intact ewes may not be stimulated by estradiol because estradiol treatment failed to increase basal LH levels prior to the surge.

Progesterone is known to influence estrous behavior in many mammals including rodents and sheep. In rodents, a progesterone surge after estrogen priming initiates estrous behavior (Edwards *et al.*, 1968). In contrast, in the ewe, progesterone priming decreases the interval from estrogen treatment to onset of estrus and increases the proportion of animals exhibiting behavioral estrus in response to estrogen (Robinson, 1954a). In the absence of progesterone priming, estrogen alone can initiate estrous behavior, however, a supraphysiological dose is required and response to estrogen diminishes when the treatment

is repeated 6 to 8 days later (Robinson, 1954b). The role of progesterone in the initiation of estrous behavior in OVX ewes treated with progesterone alone, estradiol alone or estradiol and progesterone was also examined by Karsch and coworkers (1980). Estrus was not observed in OVX control ewes receiving no steroid treatment. Increasing estradiol levels early in the simulated luteal phase (days 4-5) in ewes treated with estradiol alone, initiated estrus in all ewes at this time, however, no ewes were observed in estrus following the simulated preovulatory rise in estradiol (days 14-16). Progesterone treatment alone failed to initiate estrus in all but one ewe, which was observed in estrus between days 14 and 16. Combined treatment with estrogen and progesterone stimulated estrous behavior in all but one ewe at the appropriate time during the "artificial cycle" (days 14-16). In the non-responding ewe, a progesterone implant was broken at the time of removal and resulted in prolonged luteal phase progesterone levels and inhibition of the LH surge. Progesterone priming was required for synchrony between estrus and the LH surge because in ewes treated with estradiol alone, estrus began about 10 hr after the LH peak, whereas, treatment with both estradiol and progesterone resulted in a tight coupling (about 1 hr) between the occurrence of estrus and the LH peak.

In a more recent series of experiments, in which exogenous steroid treatments were also administered to OVX ewes to simulate natural estrous cycles (Fabre-Nys and Martin, 1991), progesterone priming was found to increase the proportion of ewes in estrus and reduce the period from rising estrogen levels to onset of estrus, however, it did not influence the magnitude of the LH surge, as was reported previously (Karsch *et al.*, 1980). Further, these researchers reported that progesterone was able to facilitate the expression of estrus in estrogen-refractive ewes (refractoriness induced by injection of 200 $\mu$g estradiol i.m. on

day 1) even when it was withdrawn 8 days before subsequent estrogen treatment (50 $\mu$g i.m. on day 19) and suggested that progesterone exerts its effect on estrous behavior by restoring estrogen sensitivity. Collectively, these data suggest that, in the ewe, increased progesterone levels during the luteal phase and the subsequent withdrawal of progesterone concomitant with rising levels of estrogen at the time of luteal regression are required for the expression and synchronization of preovulatory events such as behavioral estrus and the LH surge.

The role of endogenous ovarian steroids in regulating the preovulatory surge of LH has also been studied in cattle. Prostaglandin $F_{2\alpha}$ was used to induce luteal regression in cycling beef heifers and the secretion of progesterone, estradiol and LH was determined at various times after $PGF_{2\alpha}$ to evaluate the relationship between progesterone and estradiol secretion and the pulse frequency and amplitude of LH prior to the preovulatory surge (Imakawa *et al.*, 1986). High levels of progesterone and low levels of estradiol were observed 0 to 4 hr after $PGF_{2\alpha}$ and were associated with low LH pulse frequency ($1.6\pm0.4$ pulses/4 hr) and high LH pulse amplitude ($8.3\pm3.3$ ng/ml). In contrast, reduced serum progesterone levels (1 ng/ml) 12 to 16 hr after $PGF_{2\alpha}$, were associated with increased LH pulse frequency ($4.2\pm0.4$ pulses/4 hr) but LH pulse amplitude was not significantly altered ($4.2\pm1.0$ ng/ml). These data suggested that, in the cow, luteal phase progesterone results in low frequency LH pulses until the CL regresses at which time LH pulse frequency increases prior to ovulation. These results are supported by those of an earlier study in which the pattern of plasma LH secretion in dairy cows was found to depend upon the stage of the estrous cycle (Rahe *et al.*, 1980). Early in the luteal phase (day 3) when progesterone levels were low, LH pulses were characterized as high frequency, low amplitude, however, during the midluteal phase of the cycle (day 10 or 11) when progesterone was elevated, LH

pulses were characterized as low frequency, high amplitude. Prior to the preovulatory surge of LH, when progesterone levels had declined (day 18 or 19), LH pulses returned to the high frequency, low amplitude type observed early in the cycle.

A similar effect of progesterone on LH secretion was observed in an experiment in which subnormal levels of progesterone were maintained during the luteal phase of beef cows (Roberson *et al.*, 1989). Low levels of progesterone were maintained by administration of a progesterone-releasing intravaginal device (PRID) on day 5 followed by replacement with a new PRID on day 12 of the cycle. Corpora lutea were regressed by injection of $PGF_{2\alpha}$ on days 6, 7, and 8 to eliminate endogenous progesterone. Cows treated with PRIDs had lower circulating concentrations of progesterone ($2.14 \pm 0.29$ ng/ml) and greater frequency of LH pulses ($0.89 \pm 0.08$ pulses/hour) as compared with mean progesterone levels (6.73 ng/ml) and LH pulse frequency ($0.28 \pm 0.08$ pulses/hour) of control cows. In addition, estradiol secretion was significantly increased from days 8 to 12 and 13 to 16 as compared with control cows and the preovulatory surge occurred approximately 35 hr after PRID removal. Others have reported similar results in dairy heifers treated with PRIDs during the luteal phase of the cycle (Ireland and Roche, 1982). Collectively, these results support the concept that, in the cow, progesterone also acts in a negative manner to suppress LH pulse frequency during the luteal phase of the cycle and upon regression of the CL, progesterone negative feedback is reduced resulting in the increase in LH pulse frequency observed prior to ovulation.

Taken together, the results of experiments in sheep and cows clearly demonstrate that progesterone withdrawal at the end of the estrous cycle is the key endocrine event responsible for the changes in estradiol and LH secretion that are typically observed in these

species prior to ovulation. This is in sharp contrast to the facilitating action of progesterone on gonadotropin secretion that was described previously for rodents and that may also occur in monkeys and humans. In addition, it appears that in many mammals progesterone is also involved in the initiation of behavioral estrus and perhaps even the synchronization of female receptivity relative to the preovulatory surge of LH, although, the timing and role that it plays seems to vary among species.

In addition to steroid regulation of GnRH secretion it has been established that hypothalamic regulation of this decapeptide in laboratory rodents also includes neuromodulation by adrenergic systems, neuropeptidergic systems and EOP-producing neurons (for review, see Kalra, 1986). A potential role for adrenergic stimulation of LH secretion was demonstrated when intraventricular injections of norepinephrine (NE) and epinephrine (E) induced ovulation in the rabbit (Sawyer, 1952) and administration of adrenergic blocking agents inhibited ovulation in the rat (Sawyer,1963). Since these times there has been a plethora of research conducted, primarily in rodents, to determine the precise roles of NE and E in the initiation of the preovulatory surge of LH. Norepinephrine acts as an excitatory neurotransmitter and has been shown to increase LH secretion in a dose dependent manner (Gallo and Drouva, 1979), however, only micromolar doses of NE provoked GnRH release from steroid-treated OVX rat ME *in vitro* (Negro-Vilar and Ojeda, 1978). Crowley and coworkers (1978) demonstrated that NE activity (as measured by NE turnover) in discrete areas of the hypothalamus was increased in response to steroid-induced surges of LH. Similar increases in NE turnover were observed during proestrus in rats when LH levels were rising or had peaked (Rance *et al.*, 1981), providing further evidence that this catecholamine might be involved in evoking the preovulatory surge of LH in this

species. However, others have cautioned that the method used to determine NE turnover in these experiments was less than ideal and the assumption that the rate of NE depletion directly reflects neural activity in hypothalamic NE nerve terminals had not been validated (Kalra, 1986). There is also evidence to suggest that NE may play only a modulatory rather than obligatory role in regulating LH secretion in the rat. Reduction of hypothalamic NE content (80 to 83 percent of that in control rats) by denervation of noradrenergic input to the hypothalamus with 6-hydroxydopamine (Nicholson *et al.*, 1978) failed to alter gonadotropin secretion or disrupt the occurrence of estrous cycles and bilateral transection of the ascending noradrenergic pathway (Clifton and Sawyer, 1979) failed to completely block the occurrence of estrous cycles, which resumed 2 to 3 wk after surgery. Similar results were observed in OVX rats in which permanent disruption of the ascending noradrenergic pathway resulted in acute but not chronic alterations in LH secretion (Clifton and Steiner, 1985) or had no effect on the positive and negative feedback actions of ovarian steroids on LH secretion (Clifton and Sawyer, 1980). Collectively, these results demonstrate that NE can stimulate LH secretion but the precise role of this monoamine in the regulation of the preovulatory surge of LH is unclear.

Evidence supporting an excitatory role for epinephrine in the preovulatory surge of LH in rodents appears to be just as equivocal as that described for norepinephrine. Neuronal cell groups containing phenylethanolamine-N-methyltransferase (PNMT; converts norepinephrine → epinephrine) have been located in the posterior hypothalamus and innervate the preoptic region and other sites in the hypothalamus (Ross *et al.*, 1984). Studies utilizing the steroid primed ovariectomized rat have demonstrated that epinephrine is the most potent stimulator of LH release (Kalra and Gallo, 1983) and in the intact rat during proestrus,

epinephrine was the only catecholamine to stimulate LH secretion (Kalra, 1985). Inhibitors of PNMT activity blocked the preovulatory LH surge in intact rats (Coen and Coombs, 1983) and the steroid-induced LH surge in OVX rats (Crowley and Terry, 1981) suggesting that epinephrine is involved in LH hypersecretion. In addition, epinephrine turnover in the MBH has been reported to increase prior to the progesterone-induced LH surge in OVX rats (Adler *et al.*, 1983) and in cycling rats on the afternoon of proestrus (Mackinnon *et al.*, 1983). Hypothalamic levels of NE were severely reduced and epinephrine moderately reduced after surgical transection of brain stem projections to the hypothalamus (Brownstein *et al.*, 1976). In a similar experiment in which rats were monitored for estrus following surgery, normal estrous cycles resumed after recovery from the procedure in spite of severely reduced NE concentrations (Clifton and Sawyer, 1979) and, presumably, reduced epinephrine levels. Collectively, these data suggest that NE and E may play a modulatory role in the regulation of LH secretion because ovarian cyclicity can be maintained in the face of severely reduced, but not absence of, hypothalamic catecholamine levels. Similar effects have been reported for monkeys, in which complete deafferentation of the medial basal hypothalamus failed to alter the occurrence of menstrual cycles (Krey *et al.*, 1975; Knobil, 1980).

A recent study, however, clearly demonstrated adrenergic involvement in the regulation of GnRH pulse generator activity in OVX rats (Nishihara *et al.*, 1991). Treatment of OVX rats with either phenoxybenzamine (an $\alpha$-adrenergic receptor antagonist) or propranolol (a $\beta$-adrenergic receptor antagonist) increased the interval between MUA volleys (indicative of pulse generator activity) and inhibited the appearance of the first MUA volley after treatment for as long as 50 min. Pulses of LH were inhibited coincident with

MUA volleys after antagonist treatment. Others have reported similar results in OVX monkeys treated with $\alpha$-adrenergic antagonists (Kaufman *et al.*, 1985). Collectively, these data demonstrate adrenergic involvement in the stimulation of the GnRH pulse generator and subsequent secretion of LH in rats and monkeys but this involvement may be passive because LH secretion recovers after adrenergic denervation of the hypothalamus.

Regulation of LH secretion by neurotransmitters has also been investigated in domestic livestock (for review, see Daily *et al.*, 1987). Effects of biogenic amines, dopamine (DA), NE and serotonin (5-HT), on LH secretion in OVX ewes during the breeding season have been reported (Deaver and Daily, 1982). Infusion of OVX ewes with various concentrations of these neurotransmitters revealed differential effects on LH secretion depending on the dose administered. High concentrations of DA (66 $\mu$g/kg body weight $\cdot$ min$^{-1}$) and NE (6.6 $\mu$g/kg body weight $\cdot$ min$^{-1}$) decreased but 5-HT (6.6 $\mu$g/kg body weight $\cdot$ min$^{-1}$) increased LH secretion during the first 2 hr of infusion. Similarly, lower doses of DA (0.66 $\mu$g/kg body weight $\cdot$ min$^{-1}$) and NE (0.06 $\mu$g/kg body weight $\cdot$ min$^{-1}$) increased but 5-HT (0.06 $\mu$g/kg body weight $\cdot$ min$^{-1}$) decreased LH secretion. When ewes were injected with GnRH (25 $\mu$g, i.m.) 2 hr after the start of biogenic amine infusion, LH secretion was stimulated with all treatments but the level of response differed with dose in parallel to that described above. These data demonstrated that systemic infusion of biogenic amines could influence tonic and GnRH-induced LH secretion in the OVX ewe and that stimulatory or inhibitory effects of biogenic amines on LH secretion were dose dependent. In addition, the catecholamines (DA and NE) and serotonin had opposing effects on LH secretion.

The role of DA in the regulation of LH during the periovulatory period was examined in cycling ewes treated with $PGF_{2\alpha}$ to synchronize CL regression (Deaver and Dailey, 1983). Intravenous infusion of DA for 60 hr beginning at the time of $PGF_{2\alpha}$ treatment suppressed LH and estradiol secretion for 34 hr but did not affect the interval to the onset, magnitude or duration of the preovulatory surge of LH. Because intraventricular infusion of DA to follicular phase ewes was shown to block the LH surge (Domański *et al.*, 1975), Deaver and Daily (1983) suggested that DA may influence LH secretion at the hypothalamic level and, therefore, the systemic infusion of DA in the present experiment limited the sites of action to the ME or pituitary because it is unable to cross the blood-brain barrier. From these data it can be concluded, that in the ewe, DA can alter LH secretion but its role in the induction of the preovulatory surge of LH is not known.

Norepinephrine appears to play an important role in inducing the preovulatory surge of LH in rodents (Kalra, 1986) and a similar role has been proposed for this catecholamine in farm animals. As described previously, infusion of low doses of NE stimulated and high doses inhibited LH secretion in OVX ewes (Deaver and Daily, 1982). Norepinephrine-induced stimulation of LH was implicated in the initiation of the preovulatory surge of LH in the ewe because intraventricular infusion of an $\alpha$-adrenergic antagonist (phenoxybenzamine) on the day of proestrus inhibited ovulation (Przekop *et al.*, 1975). There are few reports in the literature in which catecholaminergic regulation of LH secretion has been examined in cattle. Treatment of prepuberal heifers with two injections, spaced 15 min apart, of NE or epinephrine (50 mg, i.m.) did not alter tonic LH secretion, however, when GnRH (100 $\mu$g, i.m.) was administered concomitant with the second injection of catecholamine, both NE and epinephrine reduced the magnitude of the LH response to

GnRH (Hardin and Randel, 1983). Similar inhibitory effects of NE and epinephrine on GnRH-induced LH secretion have been reported for OVX ewes and suggests that perhaps catecholaminergic regulation of LH secretion is similar in the ewe and cow.

More, recently the relationship between GnRH, β-endorphin, and NE secretion from the hypothalamic infundibular nuclei/median eminence (NI/ME) during the periovulatory period of the ewe was examined using perfusates collected from push-pull canulae on the day of proestrus and estrus (Domański *et al.*, 1991). Content of NE in NI/ME perfusates were low during proestrus and increased just prior to the preovulatory release of GnRH and LH and provide support for a stimulatory role of NE on LH secretion during the periovulatory period of the ewe. Collectively, these data support the concept that adrenergic (NE) systems in the ME are involved in the induction of the preovulatory surge of LH in the ewe.

Neuropeptides such as NPY and EOPs have been implicated in the control of LH secretion in rodents and have been proposed to act independently or through adrenergic neurons to regulate GnRH secretion (for review, see Kalra, 1986; Kalra and Crowley, 1992). Neuropeptide Y is a member of the pancreatic polypeptide (PP) family that is also expressed in the hypothalamus and other brain tissues of the rat (Tatemoto *et al.*, 1982; Allen *et al.*, 1983). The primary amino acid sequence of NPY consists of 36 amino acid residues and is identical in rats, guinea pigs, rabbits and humans and differs from that found in pigs and sheep by only two amino acids (Sillard *et al.*, 1989). Neuropeptide Y immunoreactivity was found in noradrenergic and adrenergic fibers in rat brain (Lundberg *et al.*, 1982; Everitt *et al.*, 1984) and the release of NE and NPY from these neurons could be differentially induced (Lundberg *et al.*, 1987). Effect of NPY on LH secretion appears to depend on prevailing steroid conditions because intraventricular injection of NPY

attenuated LH secretion in OVX rats in the absence of ovarian steroids whereas steroid priming resulted in a dose dependent stimulation in LH secretion (Kalra and Crowley, 1984). Similar results have been reported for OVX monkeys (Kaynard *et al.*, 1990) and sheep (Malven *et al.*, 1990) infused or injected with NPY in the absence of steroids. Cerebroventricular injection of ewes with NPY (13 $\mu$g) after synchronization of estrus with progesterone implants resulted in the suppression of pulsatile LH secretion for 2 hr post-treatment, however, LH secretion was not altered when GnRH was administered 15 min after NPY-treatment (McShane *et al.*, 1990). Increases in NPY concentrations in the median eminence prior to the LH surge parallel those of GnRH (Crowley *et al.*, 1985) and NPY has been demonstrated to stimulate GnRH release from median eminence explants of steroid-primed OVX rats *in vitro* (Crowley and Kalra, 1987). It appears that the mode of NPY delivery may influence the ability of this neuropeptide to regulate LH secretion because intermittent delivery, as opposed to chronic infusion, of NPY was required for stimulation of LH in steroid-primed OVX or intact rats (Kalra *et al.*, 1986). From the data presented, NPY is most likely involved in the preovulatory stimulation of GnRH secretion and hence LH release in rats, monkeys and sheep. Further, the ability of NPY to regulate LH secretion may be governed in part by the prevailing steroid milieu and the pattern of its own secretion.

It has been suggested that in addition to acting as a peptidergic neurotransmitter in the hypothalamus, to regulate GnRH secretion from the ME, NPY in the rat may also act as a neurohormone at the level of the pituitary in conjunction with GnRH to enhance LH release (Crowley *et al.*, 1986; 1987). Secretion of LH from female rat pituitary cells in culture treated with NPY alone did not differ from that of control, however, when GnRH

(1 nM) was added in the presence of varying concentrations of NPY a dose-dependent increase in LH response was observed (Crowley *et al.*, 1987). This effect has also been observed *in vivo* using pentobarbital-blocked proestrus rats (Bauer-Dantoin *et al.*, 1991). In addition, passive immunoneutralization of NPY attenuates the estrogen-induced LH surge in rats (Sutton *et al.*, 1988) providing further evidence to support a pituitary action for NPY in the initiation of the preovulatory LH surge in this species. In contrast, NPY does not appear to act directly in the pituitary in steers or ewes. *In vitro* culture of anterior pituitary cells from steers with varying concentrations of NPY (.01 to 100 nM) alone or in combination with GnRH (100 nM), did not result in a significant increase in basal or GnRH-induced LH secretion (Chao *et al.*, 1987). Similarly, NPY ($10^{-10}$ to $10^{-6}$ M) alone or in conjunction with GnRH had no effect on LH secretion from ovine pituitary cells *in vitro* either in the presence or absence of estradiol (Brooks *et al.*, 1991). Collectively, these data suggest that there are species differences in the pituitary action of NPY, however, it seems likely that in rodents, monkeys and sheep, and perhaps other mammals, hypothalamic GnRH secretion is regulated by NPY.

As with other neurotransmitters and peptide hormones, NPY mediates its effects through specific membrane receptors and using autoradiographic techniques NPY binding has been demonstrated in the hypothalamus and ME of rats (Lynch *et al.*, 1989). Biochemical analysis of receptor binding has suggested that there are two distinct NPY receptor subtypes, $Y_1$ and $Y_2$ in rat brain (Walker and Miller, 1989). The mechanism through which NPY exerts its effects appears to differ for each receptor subtype. The $Y_1$ receptor is coupled to phosphoinositide hydrolysis, intracellular mobilization of calcium and reduction in adenylate cyclase activity (Aakerlund *et al.*, 1990) whereas NPY binding to the

$Y_2$ receptor results in the reduction of calcium influx through voltage-dependent channels (Walker *et al.*, 1988). Both the excitatory and inhibitory actions of neuropeptide Y are believed to be mediated through the $Y_1$ receptor because administration of [$Leu^{31}$, $Pro^{34}$]NPY ($Y_1$ receptor agonist) provoked LH release in steroid-primed rats and inhibited LH secretion in OVX rats not treated with steroids (Fuhlendorff *et al.*, 1990). Similar treatment with $NPY_{13-16}$ ($Y_2$ receptor agonist) failed to alter LH secretion in either experimental model (Wahlestedt *et al.*, 1986).

Neuropeptide Y levels in the median eminence and hypophysial portal blood fluctuate with increased secretion of LH and the LH surge in rats on the day of proestrus (Sahu *et al.*, 1989) and it has been suggested that NPY secretory dynamics may be regulated by ovarian steroids because estrogen treatment of OVX rats suppressed and progesterone enhanced ME NPY levels (Crowley *et al.*, 1985). Further, NPY levels fluctuate in the rat arcuate nucleus, an area where both estrogen-concentrating and NPY-producing neurons are colocalized (Chronwall *et al.*, 1985). More recently, colocalization of nuclear $^3$H-estradiol and cytoplasmic NPY immunoreactivity was described in 10-20% of the neurons in this region of the rat hypothalamus (Sar *et al.*, 1990). In light of the importance of steroids in the regulation of the preovulatory surge of LH, it is not surprising that ovarian steroids are involved in the regulation of NPY secretion in the hypothalamus and, in fact, further strengthens the supposition that this neuropeptide is an important regulatory factor in GnRH and LH secretion.

Endogenous opioid peptides have received considerable attention in reproductive research endeavors over the past 20 years and the peptidergic neurons that produce them have been recognized to play an important inhibitory role in the regulation of LH secretion

in many mammals, including laboratory rodents, primates and domestic livestock. The neuroregulatory action of opioids on LH secretion in rats, primates and domestic livestock has been reviewed (Kalra, 1986; Malven, 1986; Ferin, 1987; Haynes *et al.*, 1989; Barb *et al.*, 1991). Three classes of EOP have been isolated and characterized: **enkephalins**, **β-endorphins**, and **dynorphins**. Two opioid pentapeptides, methionine-enkephalin (Met-ENK; Tyr-Gly-Gly-Phe-Met) and leucine-enkephalin (Leu-ENK; Tyr-Gly-Gly-Phe-Leu), were first isolated from pig brain (Hughes, 1975) and the cDNA for bovine adrenal pro-enkephalin has been cloned and sequenced (Noda *et al.*, 1982). In the rat, enkephalins are produced predominantly by interneurons in the brain and medulla (Fallon and Leslie, 1986). Immunoreactivity to pro-enkephalin has been colocalized with that of oxytocin in bovine hypothalamus (Vanderhaeghen *et al.*, 1983).

The 31 amino acid sequence of β-endorphin was first reported by two laboratories in 1976 (Bradbury *et al.*, 1976; Li and Chung, 1976) and subsequently it was determined that β-endorphin is the major opioid cleavage product of a larger precursor molecule (241 amino acids), pre-pro-opiomelanocortin (POMC), that can be differentially cleaved to produce a number of peptide hormones. Pre-pro-opiomelanocortin is synthesized in rat anterior pituitary (Mains *et al.*, 1977) as well as neuronal cell bodies in the hypothalamus (arcuate nucleus) of rats (Bugnon *et al.*, 1979), pigs (Kineman *et al.*, 1988), sheep (Nilaver *et al.*, 1979) and cows (Leshin *et al.*, 1988). High concentrations of β-endorphins are found in the anterior and intermediate lobes of porcine (Smyth and Zakarian, 1980) and bovine (Crine *et al.*, 1977) pituitaries. Peptide derivatives of POMC are also present in monkey arcuate nucleus, anterior pituitary and intermediate lobe (Ferin, 1987).

Pro-dynorphin consists of 234 amino acids and cleavage of this precursor molecule results in several opioid peptides that are extensions of leu-enkephalin: $\alpha$- neo-endorphin and dynorphins A and B (Akil *et al.*, 1984). Dynorphin A was first isolated from porcine pituitaries and was found to consist of 17 amino acid residues (Goldstein *et al.*, 1981). In the rat, pro-dynorphin is synthesized in pituitary gonadotrophs (Khatchaturian *et al.*, 1986) as well as neurons in the paraventricular nucleus (Palkovits *et al.*, 1983). Immunoreactive dynorphin A has been detected in bovine anterior pituitary, neural intermediate lobe, hypothalamus and stalk median eminence (Chao and Malven, 1987) and ovine anterior pituitary (Chao *et al.*, 1987). However, in pigs (Fischli *et al.*, 1982), rats (Day and Akil, 1986), cattle (Chao and Malven, 1987) and sheep (Chao *et al.*, 1987) a larger molecular weight form of dynorphin A is found in the anterior pituitary while smaller forms exist in the neural intermediate lobe. It has been suggested that the larger forms of dynorphin may serve as degradation resistant precursors that are cleaved into smaller bioactive forms only at a time of physiological need (Chao and Malven, 1987).

Because there are a variety of EOPs, it is not surprising that there are multiple opioid receptor subtypes designated $\mu$, $\delta$, $\kappa$, $\sigma$, and $\epsilon$. The receptor subtypes are not identical and although all EOPs, at high enough concentrations, demonstrate binding affinity to more than one receptor subtype, each class of EOP exhibits binding preference to one type of receptor over the others. In general enkephalins prefer binding to $\delta$, $\beta$-endorphin to $\mu$, and dynorphins to $\kappa$ receptors (Paterson *et al.*, 1984). Opioid receptor distribution appears to parallel that of the opioid producing cells and opioid receptor binding has been demonstrated in the hypothalamus of rats (Leadem *et al.*, 1985), monkeys (Lewis *et al.*, 1984), cows (Leshin *et al.*, 1991) and sheep (Yang *et al.*, 1989a). Low levels of opiate receptors are

present in the anterior pituitary of the rat (Simantov and Snyder, 1977) but not in the monkey (Wamsley, 1982) or the ewe (Horton *et al.*, 1990). Collectively these data demonstrate that, in several mammals, opioid peptidergic neurons and receptors are located in the hypothalamus in proximity to GnRH neurons and are, therefore, in a good position, both anatomically and physiologically, to influence GnRH secretion.

Intraventricular injection of castrated rats with β-endorphin (Kinoshita *et al.*, 1980) or dynorphin (Kinoshita *et al.*, 1982) suppressed LH secretion within 30 min, however, the effect was less pronounced in dynorphin-treated rats. Similar treatment of OVX rats with Leu-ENK stimulated LH secretion whereas Met-ENK was without effect (Leadem and Kalra, 1985a). Clearly, these data demonstrate that these three classes of EOPs are capable of altering LH secretion in the rat. The effect of increased opioid tone on LH secretion has been examined in OVX rats (Leadem and Kalra, 1985a). Intraventricular infusion of β-endorphin (10 μg/hour) resulted in severe attenuation of pulsatile LH secretion for 3 hr after treatment and led these investigators to hypothesize that the endogenous release of β-endorphin near GnRH neurons could evoke a similar response. In cyclic rats, the LH surge and ovulation were blocked by intraventricular injections of β-endorphin and the blockade reversed by administration of naloxone (Leadem and Kalra, 1985b). Collectively, these data suggest that GnRH secretory patterns may be regulated by altering the degree of inhibitory opioid tone.

Further evidence that inhibitory opioid tone restrains GnRH and LH secretion has been provided by *in vitro* and *in vivo* experiments in which the opioid **antagonist** naloxone was infused to reduce EOP tone. Infusion of steroid-primed OVX rat MBH-POA with naloxone *in vitro* elicited a rapid release of GnRH (Leadem *et al.*, 1985) and *in vivo* infusion

of naloxone (2 mg/hour) to intact rats prior to the LH surge on the day of proestrus increased LH pulse frequency and amplitude and produced a surge of LH that closely resembled that normally observed in intact rats on the afternoon of proestrus (Kalra, 1986). Data from the experiments described above, suggest that reduction of hypothalamic opioid tone on the day of proestrus is an important factor in the events leading to the preovulatory LH surge in the rat.

A functional link between adrenergic neurons and opioid receptor mediated regulation of GnRH secretion was demonstrated in steroid-primed OVX rats. Blocking $\alpha$-adrenergic receptors with phenoxybenzamine (an $\alpha$-adrenergic receptor antagonist) or decreasing hypothalamic norepinephrine and epinephrine levels with dopamine-$\beta$-hydroxylase (DBH) inhibitors prior to treatment with naloxone resulted in an inhibition of the naloxone-induced release of LH that is normally evoked by infusion of this opioid antagonist. In addition treatment with either a dopamine agonist (apomorphine) or antagonist (pimozide) had no effect on LH secretion in response to naloxone (Kalra and Simpkins, 1981). Collectively, these data suggest that EOP neurons may exert their effects on GnRH neurons, and hence GnRH and LH secretion, via adrenergic, but not dopaminergic, systems in the rat hypothalamus (Kalra, 1986). Further evidence in support of this hypothesis was provided by Nishihara and coworkers (1991). These investigators reported that administration of naloxone (2 mg/kg body weight, i.v.), to ovariectomized rats 10 min after an injection of propranolol (5 mg/kg body weight, i.v.), prevented propranolol-induced attenuation of MUA volleys and LH pulses in medial basal hypothalamus and peripheral circulation, respectively.

Not surprisingly, endogenous opioid peptides also appear to be involved in the regulation of gonadotropin secretion in both sheep and cows. Several reports of the

suppressive effects of endogenous opioids as well as opioid agonists have been reported for both species. Intracerebroventricular administration of β-endorphin (10 μg) decreased LH pulse frequency in OVX and follicular phase, but not luteal phase, cyclic ewes (Horton *et al.*, 1989). Immunoneutralization of endogenous β-endorphin and Met-ENK in luteal phase ewes by intracerebral infusion of specific antisera directed against ovine β-endorphin or Met-ENK resulted in abrupt increases in LH secretion within 10-20 min of infusion (Weesner and Malven, 1990). Similarly, Short and coworkers (1987) reported that injection of an opioid agonist (bremazocine; 0.45 mg, i.v.) every 15 min for 6 hr to heifers during the follicular phase of the cycle reduced both LH pulse frequency and amplitude. Armstrong and Johnson (1989) reported similar suppressive effects of opioid agonists, [D-Ala$^2$,Me,Phe$^4$,-Met(0)ol]-enkephalin and morphine, on episodic LH secretion in beef heifers injected during the follicular phase of the cycle. Collectively these data indicate that endogenous opioids may act to suppress LH secretion during the follicular phase of ewes and cows and perhaps the luteal phase of ewes.

A large body of research has accumulated in which the opioid antagonists naloxone and WIN 44441-3 (WIN) were found to stimulate LH secretion in sheep and cows. The level of opioid inhibition, as assessed by LH response to opioid antagonist treatment, appears to vary depending on steroid milieu present at the time of treatment. It is generally agreed that in the ewe systemic administration of opioid antagonists during the luteal phase of the cycle increases systemic concentrations of LH by increasing LH pulse frequency (Brooks *et al.*, 1986b; Whisnant and Goodman, 1988). However, there are number of conflicting reports concerning the effect of opioid antagonists administered during the follicular phase of the cycle. Systemic (Brooks *et al.*, 1986b) or intracerebroventricular (Horton *et al.*,

1989) administration of naloxone to follicular phase ewes increased plasma concentrations of LH by increasing the pulse frequency of this gonadotropin. In contrast, others have reported no increase in LH secretion (Malven *et al.*, 1984), increase in pulse amplitude and decrease in pulse frequency (Currie and Rawlings, 1987) and increase in LH pulse amplitude with no effect on frequency (Whisnant *et al.*, 1988) after opioid antagonist (naloxone or WIN) treatment of follicular phase ewes. The disparity in results between experiments conducted during the follicular phase is not clear but may be related to differences in the EOP antagonist used or the class of opioid receptor that binds the antagonist. Both naloxone and WIN can bind to $\mu$, $\delta$ and $\kappa$ receptor subtypes but WIN has a higher binding affinity for $\kappa$ receptors than naloxone (Whisnant and Goodman, 1988) and naloxone has greater affinity for the $\mu$ and $\delta$ receptors (Chang, 1984). Collectively, these results suggest that opioids are involved in the regulation of LH during both the follicular and luteal phase of the estrous cycle of the ewe and that the prevailing steroid milieu may influence the level of opioid tone present during the various phases of the cycle.

Few experiments have been conducted to evaluate the effects of opiate antagonists during the estrous cycle of the cow. It appears that cows respond similarly to ewes when opioid antagonists are administered during the follicular phase of the cycle because treatment of follicular phase heifers with the opioid antagonist quadazocine (WIN 44441-3; 210 mg, i.v.) every 2 hr for 6 hr stimulated an increase in LH secretion, however, only pulse amplitude and not pulse frequency was increased. Contrary to the case in the ewe, administration of the antagonist to luteal phase heifers (day 13 of the cycle) had no effect on LH secretory patterns (Short *et al.*, 1987). These data suggest that in the cow, opioids may be involved in the modulation of LH secretion during the follicular but not luteal phase

of the cycle, however, more research is necessary in order to determine the exact role that endogenous opioids play in the regulation of LH secretion during the various stages of the estrous cycle of domestic species. In addition, because decreasing opioid tone during the luteal phase of the cycle resulted in enhanced LH secretion in the ewe but not the cow, species specific differences may exist among domestic livestock with respect to opioidergic regulation of LH secretion.

Because a decrease in opioid tone during the periovulatory period has been proposed to allow the preovulatory gonadotropin surge to occur in rats (Kalra, 1986) and humans (Blankstein *et al.*, 1981) it was of interest to determine if a similar mechanism occurred in livestock. An experiment was conducted to determine if reduced EOP activity during the follicular phase of the estrous cycle would permit the preovulatory LH surge to occur in ewes (Currie *et al.*, 1991). Estrus was synchronized by treatment for 12 days with intravaginal sponges releasing medroxyprogesterone acetate. Upon sponge removal, saline, naloxone or morphine was injected hourly via jugular canulae to maintain increased levels of naloxone or morphine in the circulation during the follicular phase, estrus and at the time of the gonadotropin surge. Jugular blood samples were collected hourly prior to each injection and every 6 hr for 72 hr after the last injection for quantification of LH. Reducing opioid tone during the periovulatory period with naloxone had no effect on the interval from sponge removal to the gonadotropin surge ($52 \pm 4.4$ hour) as compared with saline-treated control ewes ($56 \pm 5.4$ hour), however, similar treatment with morphine, to increase opioid tone, significantly delayed the occurrence of estrus ($73 \pm 10.5$ hour). In addition, neither tonic LH nor peak LH concentrations were affected by naloxone or morphine treatment. Because naloxone injection did not alter tonic LH secretion or the period from sponge

removal to the occurrence of estrus and the preovulatory LH surge these authors suggested that LH secretion is not tonically suppressed by opioid peptide activity at naloxone sensitive receptors during the follicular phase or preovulatory surge of LH.

In several additional experiments (Currie *et al.*, 1991), ewes treated with morphine from 24 to 48 or from 18 to 30 hr after sponge removal had suppressed tonic LH secretion, however, when morphine was administered from 24 to 36 after sponge removal no effect on tonic LH secretion was observed. These data suggest that opioid peptides can suppress LH secretion during the periovulatory period of the ewe, however, the failure of morphine to consistently suppress tonic LH secretion at various times after progestogen withdrawal in these experiments was unexpected and could not be explained. Because, in the first experiment, infusion of morphine delayed the onset to estrus and the preovulatory surge of LH but did not block ovulation, it was proposed that withdrawal of opioid tone following the luteal phase in sheep may allow the occurrence of the LH surge, however, reduction of opioid activity at morphine sensitive receptors may not be critical for the occurrence of estrus or the preovulatory surge of gonadotropins.

Circumstantial evidence linking opioid, noradrenergic and GnRH systems to the preovulatory surge of LH in ewes has been reported (Domański *et al.*, 1991). Release of GnRH, $\beta$-endorphin and norepinephrine from the infundibular nuclei/median eminence (NI/ME) during proestrus and estrus were quantified in perfusates collected from the NI/ME using push-pull cannulae. Concentration of GnRH in perfusates was low on the day of proestrus but increased and was maximal on the day of estrus. Plasma levels of LH increased late in proestrus and on the day of estrus prior to the LH surge. In addition, the preovulatory surge of LH coincided with maximal GnRH release from the NI/ME on the day

of estrus. Conversely, β-endorphin secretion from NI/ME increased on the day of proestrus and was maximal late in proestrus and then declined and remained low on the day of estrus. Changes in plasma levels of β-endorphin in the peripheral circulation on the day of proestrus and estrus occurred in parallel with those observed in the NI/ME perfusates. Finally, NE concentration in NI/ME perfusates were low during proestrus and peaked shortly before the GnRH and LH surges on the day of estrus. Collectively these observations have led Domański and coworkers (1991) to suggest a sequence of neuroendocrine events, in the ewe, that begins on the day of proestrus and culminates in the preovulatory surge of LH on the day of estrus: (1) Increased secretion of β-endorphin on the day of proestrus inhibits GnRH secretion thereby increasing the releasable pool of this decapeptide in the NI/ME; (2) Decreasing secretion of β-endorphin on the day of estrus allows NE activity to increase and this increase in NE tone facilitates the release of accumulated GnRH; (3) Increased GnRH output triggers the preovulatory surge of LH. Although this model for the regulation of periovulatory LH secretion in the ewe can account for the observed changes in LH secretion at this time, the involvement of neuropeptide Y in this scheme of events was not addressed. A similar sequence of neuroendocrine events has been proposed to occur during proestrus and estrus in rats (Kalra, 1986).

Although it appears that EOPs inhibit episodic LH secretion during both the follicular and luteal phases of the ovine estrous cycle, the site of opioid action remained unknown because in most cases opioid antagonists were administered systemically. Recently several experiments have investigated possible sites of opioid action in the hypothalamus (Malven *et al.*, 1990; Whisnant *et al.*, 1991) and pituitary (Horton *et al.*, 1990) of the ewe. Opioids most likely exert their effects at the level of the hypothalamus in the ewe because $^{3}$H-

naloxone bound specifically to hypothalamic membranes (160-184 fmol/mg protein) but not anterior pituitary membranes *in vitro* (Horton *et al.*, 1990). Using stereotaxically implanted intracerebral guide tubes in various brain sites of luteal phase ewes, Malven and coworkers (1990) determined that intracerebral infusion of naloxone to the basal forebrain and chiasmatic areas consistently provoked an increase in LH secretion whereas infusion of naloxone to the anterior, ventromedial and lateral hypothalamic area, including the arcuate nucleus and third ventricle, failed to consistently stimulate LH secretion. Naloxone sensitive sites appeared to form a continuum from the ventrolateral septum, diagonal band of Brocca and nucleus accumbens into the preoptic area in and around the organum vasculosum of the lamina terminalis. It has been reported that 50 % of GnRH perikarya in the ewe are localized in the area surrounding the organum vasculosum (Caldani *et al.*, 1988) which coincides with the location of naloxone sensitive sites.

Using a slightly different approach, Whisnant and coworkers (1991) stereotaxically placed WIN implants into the preoptic area (POA) and MBH of cycling ewes. Jugular blood samples were collected during the luteal (days 7-8) or follicular (24 hr after $PGF_{2\alpha}$ injection) phases of the cycle. During the luteal phase of the cycle WIN implants increased LH pulse frequency but not amplitude in both the POA and MBH. Interestingly, during the follicular phase WIN implants increased LH pulse frequency but not amplitude in the POA and LH pulse amplitude but not frequency in the MBH. These data suggest that, while opioids act in both the POA and MBH to suppress LH secretion, different populations of EOP neurons may be stimulated depending on the phase of the estrous cycle. Based on these data and others (Whisnant and Goodman, 1988) these investigators have hypothesized that in the ewe, the estrogen-induced decrease in LH pulse amplitude observed during the follicular phase

may be mediated by opioidergic neurons acting in the MBH. In contrast, progesterone-induced reduction in LH pulse frequency observed during the luteal phase may be conducted by EOP neurons acting at both the POA and MBH.

Sites of opioid inhibition of GnRH secretion in the cow have been investigated by Leshin and coworkers (1991). Opioid receptors in the ME and POA of beef heifer brains were quantified by autoradiography using $^{3}$H-naloxone as the receptor ligand. In addition, ME and POA tissue from dairy and beef cows were perfused *in vitro* with naloxone to examine the effect of this opioid antagonist on GnRH secretion from these two regions of the brain. Specific binding of $^{3}$H-naloxone was observed in both tissues and autoradiographic analysis of specific naloxone binding sites revealed similar mean binding site densities in the ME ($67.5 \pm 8.0$ fmol/mm$^2$ tissue) and POA ($80.3 \pm 5.8$ fmol/mm$^2$ tissue) regions of bovine brain. Treatment of perfused ME and POA halves with naloxone resulted in enhanced release of GnRH from both tissues. Therefore, these data implicate the ME and POA as potential sites for the opioidergic regulation of GnRH secretion in this species.

It should be noted that evidence is accumulating to support a role for excitatory amino acids (EAA) in the regulation of gonadotropin secretion in some species. Early reports indicated that administration of excitatory amino acids could stimulate LH secretion in rats (Ondo *et al.*, 1976) and monkeys (Wilson and Knobil, 1982) and suggested that endogenous EAA might be involved in the regulation of LH secretion in these species. Although there are several EAA that may function as neurotransmitters, L-glutamate is thought to be the primary mediator of EAA action in the central nervous system (for review, see Shank and Aprison, 1988; Nicoll *et al.*, 1990). Several subtypes of EAA receptors have been characterized based upon their activation by specific EAA agonists. Of the various

receptor subtypes, the receptor activated by N-methyl-D-aspartate (NMDA) has been most clearly defined (Watkins and Evans, 1981). Administration of competitive and noncompetitive NMDA antagonists to OVX rats *in vivo* blocked the estrogen-induced surge of LH (López *et al.*, 1990; Urbanski and Ojeda, 1990). A noncompetitive NMDA receptor antagonist blocked the progesterone-induced LH surge in estrogen-primed OVX rats as well as ovulation and the LH surge in intact cycling rats treated on the day of proestrus (Brann and Mahesh, 1991b). The effects of EAA on LH secretion appear to be mediated at the level of the hypothalamus and not the pituitary because EAA stimulated GnRH release from rat arcuate nucleus-median eminence fragments (López *et al.*, 1992) but failed to alter basal or GnRH-induced LH secretion from rat or monkey pituitaries (Tal *et al.*, 1983) incubated *in vitro*. Further, when arcuate nucleus-median eminence fragments from male rats were incubated with various glutamate agonists *in vitro* EAA-induced GnRH release was mediated primarily through non-NMDA receptors (at lower ligand concentrations) although NMDA receptors appeared to mediate GnRH release in response to certain endogenous agonists (López *et al.*, 1992). Collectively, these data suggest that EAA may be involved in the preovulatory surge of LH in the rat, and perhaps the monkey, and that these neurotransmitters mediate their effects through a suprapituitary mechanism.

The preovulatory surge of LH in mammals is a complex event that involves the neuroendocrine regulation of a network of neural pathways in the hypothalamus that is coordinated with hormonal regulation of biochemical processes in the anterior pituitary. From the data presented, it seems likely that at least four neuronal systems are involved in the regulation of the preovulatory surge of LH in several mammalian species, including the ewe and cow. These systems are the steroid concentrating neurons, adrenergic networks,

and NPY-and EOP-producing neurons. In addition it now appears that EAA may also be involved in the induction of the preovulatory surge of LH in some species. The functional and anatomical links of the neuroendocrine apparatus that generates the LH surge are quite complex but, generally, it appears that the changing steroid milieu (decreasing progesterone and increasing estradiol) during proestrus is associated with decreasing opioid tone, which in turn results in increased secretion of adrenergic transmitters (epinephrine and norepinephrine) and NPY in the hypothalamus. These neuromodulators, and perhaps EAA, act in concert to increase GnRH secretion, which in turn provokes the preovulatory surge of LH. In addition estradiol, and NPY in some species, may increase pituitary sensitivity to GnRH, thereby facilitating LH synthesis and release.

**Developmental and Functional Aspects of the Corpus Luteum**

The corpus luteum is an ephemeral endocrine organ that is formed after ovulation from cells that line the ovulatory follicle (for review, see Niswender *et al.*, 1985; Auletta and Flint, 1988; Niswender and Nett, 1988). The life span of the CL determines the length of the estrous cycle of a number of mammalian species. As described previously, the CL develops after ovulation and attains maximal size and progesterone production midcycle. Progesterone secretion remains elevated until late in the cycle and then decreases rapidly as the CL begins to regress. Declining systemic concentrations of progesterone in concert with increased secretion of estrogen from developing follicles initiates behavioral estrus or a new cycle. In a majority of species, should pregnancy occur, the CL of the cycle is maintained (does not regress) throughout gestation and ovarian cycles cease. It is generally accepted that the primary function of the CL is to synthesize and secrete progesterone, which prepares

the uterine endometrium for pregnancy. Luteinizing hormone is considered the primary luteotropin in many species because luteal progesterone production is maintained by this gonadotropin during the estrous or menstrual cycle. It also should be noted that, although progesterone is the primary hormone synthesized and secreted by the CL, estrogen, $PGF_{2\alpha}$, relaxin and oxytocin are also produced by luteal cells in a variety of species; however, the specific hormone(s) synthesized, in addition to progesterone, varies among species (for references, see Niswender and Nett, 1988).

***Large and Small Luteal cells***

The origin of luteal cells has been of interest since the early twentieth century when Loeb (1906) proposed that the CL of the guinea pig consisted of cells derived from both granulosa and theca layers of the follicle. It is now clear that CL of monkeys (Corner, 1945), rats (Pedersen, 1951), pigs (Corner, 1919), sheep (O'Shea *et al.*, 1980) and cattle (Donaldson and Hansel, 1965a) are similarly formed. Steroidogenic luteal cells from sheep (Fitz *et al.*, 1982) and cows (Koos and Hansel, 1981) and other species (Niswender and Nett, 1988) occur as two distinct populations of smaller and larger cells commonly referred to as **small luteal cells (SLC)** and **large luteal cells (LLC)**. Because luteal tissue from cows and sheep can be obtained with relative ease and in considerable quantity, the morphological and functional aspects of large and small luteal cells from these species have been very well characterized.

Donaldson and Hansel (1965a) described the early formation, subsequent growth and demise of the bovine CL as assessed from detailed histological analysis of CL and follicles collected from cows at various stages of the estrous cycle. These investigators reported that luteinization of both granulosa and theca cells of the preovulatory follicle occurred as early

as 6 hr after the onset of estrus; prior to ovulation. Mitotic activity was observed more frequently in granulosa as compared with theca cells prior to ovulation. After ovulation, the granulosa and theca layers became deeply folded as the follicle wall collapsed. Between 24 and 48 hr after ovulation mitotic activity was observed in luteal, connective tissue and endothelial cells and the developing CL appeared to be composed of small and medium luteal cells at this time. Early in the cycle (days 3 and 4), SLC were localized around connective tissue trabeculae (of theca origin) in the center of the CL whereas LLC were most numerous in areas distant from the trabeculae. Mitotic activity was exclusively localized to SLC and connective tissue cells in and around the trabeculae. On day 7 of the cycle, the distribution of small, medium and large cells within the connective tissue reticulum was more homogeneous. Large luteal cells were usually associated with SLC, blood vessels and lymphatics, however, small luteal cells were not always associated with LLC and at this stage of the cycle, mitotic activity was confined to small cells near the trabeculae. At midcycle (days 9 to 11), mitotic activity was limited to connective tissue cells and early degenerative changes suggestive of decreased hormone production (decreased cytoplasmic stippling, rounding of cell outline and vacuolation near periphery of the cell) were observed in LLC. Arteriole walls were thickened at this stage of the cycle and became completely occluded in the regressing CL (day 21). As the CL regressed, signs of further degeneration of LLC were observed that included condensation of the cytoplasm, less pronounced nucleoli and pycnotic nuclei. Corpora lutea collected on day 21 contained some non-stippled LLC, however, no SLC were observed.

Because CL size increased threefold from days 4 to 7 when mitotic activity occurred mostly in SLC, Donaldson and Hansel (1965a) proposed that SLC enlarged to form LLC and

that the medium cells observed were SLC in the process of differentiating into LLC. In addition, because progesterone content ($\mu$g/CL) increased with no concomitant increase in progesterone concentration ($\mu$g/g tissue) between days 4 and 7 of the cycle, these researchers speculated that the increase in progesterone content was due to an increase in secretory cell numbers as a result of the transformation of SLC into LLC. Additional studies with bLH and hCG suggested that gonadotropins may stimulate the transformation of SLC to LLC because CL (day 7) from cows treated with either bLH or hCG early in the cycle were larger and had significantly increased concentrations of progesterone, which could be explained by an increase in the number and secretion rate of large luteal cells. Histological evaluation of these CL revealed an abnormal association of luteal cell types as compared with CL from control cows and in many respects appeared to resemble actively growing CL early in the cycle (day 4). Transformation of SLC to LLC during the estrous cycle has also been reported for untreated ewes (Schwall *et al.*, 1986) and ewes treated with either LH or hCG between days 5 to 10 of the cycle (Farin *et al.*, 1988).

More recently, using specific monoclonal antibodies against bovine granulosa (GrAb) or theca (TAb) cell surface antigens, Alila and Hansel (1984) provided evidence that strongly supported the original hypothesis of Donaldson and Hansel (1965a) concerning the origin of SLC and LLC. Results of these studies demonstrated that a majority of SLC bound TAb and the percentage of small cells binding this antibody remained relatively constant during the cycle. In contrast, GrAb binding was evident in only 14 percent of small cells and then only between days 4 and 6 of the cycle. Significant changes in the percentage of LLC binding TAb or GrAb were observed over the course of the cycle. The percentage of LLC that bound GrAb was greatest early in the cycle (days 4 to 6) and declined thereafter,

however, the proportion of LLC binding TAb increased from days 4 to 6 and 10 to 12, remaining unchanged from days 16 to 18. Data from this study provide further evidence to support the hypothesis that SLC and LLC originate from theca and granulosa cells, respectively, and because TAb bound to both LLC and SLC between days 10 to 12 of the cycle it seems likely that SLC develop into LLC as the CL ages. Collectively, these data suggest that the growth and development of the CL and associated changes in cellular composition during the estrous cycle are dynamic processes that are regulated by LH.

Morphological and ultrastructural differences between SLC and LLC from sheep and cattle have been summarized in several recent reviews (Niswender *et al.*, 1985; Niswender and Nett, 1988; Farin *et al.*, 1989; Hansel *et al.*, 1991). Small luteal cells are 22 $\mu$m or less in diameter and are spindle-shaped in appearance. These cells are further characterized by an irregular-shaped nucleus with cytoplasmic inclusions or invaginations and dark staining cytoplasm that contains large lipid droplets. Small cells contain a moderate number of mitochondria that may appear round, elongated or branching in shape and contain tubular or lamelliform cristae. In addition, SLC contain large amounts of smooth, tubular endoplasmic reticulum characteristic of steroid secreting cells. Conspicuously absent are membrane bound secretory granules that are abundant in the cytoplasm of the LLC.

Minimum acceptable diameter for classification as a LLC varies among studies and ranges from 23 to 26 $\mu$m. Large cells are polyhedral in shape with a light staining cytoplasm and large, centrally located nucleus with prominent nucleoli. These cells contain numerous mitochondria that may be spherical, cup-shaped or elongated in form and contain predominantly tubular cristae. In the peripheral regions of the cell lies an abundance of smooth endoplasmic reticulum in the form of branched tubules, tubular sheets and

fenestrated cisternae. In addition, an extensive Golgi complex is located to one side of the nucleus and, in contrast to the SLC, the cytoplasm of the LLC has few lipid droplets and contains rough endoplasmic reticulum and numerous membrane bound secretory granules. These secretory granules contain oxytocin and in some species, relaxin.

Ultrastructural morphometric analysis of the cellular composition of bovine CL (approximately day 12), after synchronization with a $PGF_{2\alpha}$ analog, revealed that LLC represented only 3.5 percent of all cells within luteal tissue (O'Shea *et al.*, 1989). Endothelial cells/pericytes were the most numerous (52.3%) followed by SLC (26.7%), fibrocytes (10.0%) and other cell types (7.5%). Therefore, SLC outnumbered LLC in luteal tissue by a ratio of 7.6:1. However, based upon point-count measurements of volume density, LLC occupied the greatest percentage of luteal tissue (40.2±7%) followed by SLC (27.7±6.3%) and endothelial cells/pericytes (13.3±1.7); the remainder occupied by intercellular space, fibrocytes, other cell types and vessel lumens. Others, using enzymatic dispersion methods to determine cell numbers, have reported that SLC outnumber LLC in the bovine CL (days 10 to 12) by a ratio of 20:1-40:1 (Hansel *et al.*, 1987). The discrepancy between the two studies may be due to the prostaglandin analog treatment employed by O'Shea and coworkers (1989) to synchronize the occurrence of estrus. Corpora lutea (day 13) from Brahman cows synchronized with a $PGF_{2\alpha}$ analog had fewer numbers of small and large luteal cells and decreased progesterone secretion (days 3 to 12) compared with CL from untreated control cows (Hansen *et al.*, 1987) suggesting that CL formed after synchronization with $PGF_{2\alpha}$ and CL from natural cycles may not be structurally or functionally equivalent. On the other hand, O'Shea and coworkers suggested that the use of enzymatic dispersion to dissociate luteal cells (Hansel *et al.*, 1987) may preferentially

destroy LLC, resulting in an underestimation of LLC numbers. Irrespective of the method used to determine luteal cell numbers, it seems clear that there are more SLC than LLC in the bovine CL at midcycle.

Similar results have been reported for the ewe in which changes in steroidogenic luteal cell populations were determined throughout the estrous cycle (Farin *et al.*, 1986). Numbers of SLC and LLC were similar on day 4 of the cycle, however, during the latter half of the cycle (days 8 to 16) SLC were observed to outnumber LLC. Further, these investigators reported that between days 4 and 12, LLC increased threefold in size with no corresponding increase in cell number whereas the number of SLC increased fourfold between days 4 and 8 but with no increase in cell size over the duration of the estrous cycle. It should be noted, however, that the latter finding, in which the number of LLC remained constant throughout the cycle, is at odds with the hypothesis of Donaldson and Hansel (1965a) and Farin and coworkers (1988) in which LH was proposed to stimulate the conversion of SLC to LLC. These data also conflict with those from an earlier report (Schwall *et al.*, 1986) from the same laboratory as Farin and colleagues (1986) in which the number of LLC from CL of untreated ewes increased between days 4 to 8 whereas the number of SLC increased between days 8 to 12 of the estrous cycle. The ratio of large to small cells increased linearly from day 4 to 16 of the cycle and the increase observed early in the cycle appeared to be due to an increase in cell size whereas that observed later in the cycle was attributed to a loss of small cells. Others have reported similar large to small cell ratios in CL from ewes in midcycle (O'Shea *et al.*, 1979; Rodgers *et al.*, 1984) despite the use of different methods. The reason for the disparity between the studies is not clear, however, it does appear that LLC from ovine CL increase in number during the first half

of the estrous cycle as has been reported for bovine CL (Donaldson and Hansel, 1965a).

In addition to size and morphology, small and large luteal cells can also be distinguished biochemically by differences in various receptor populations on their cell surfaces. The distribution of LH, $PGE_2$ and $PGF_{2\alpha}$ receptors were reported to differ between large and small luteal cells isolated from CL of superovulated ewes (Fitz *et al.*, 1982). Corpora lutea were collected between days 8 to 12 after treatment with hCG and subjected to enzymatic dispersion followed by elutriation, which resulted in enriched populations of small and large cells. Quantification of specific binding sites for hCG revealed that SLC had significantly more LH/hCG receptors than did LLC. In contrast, LLC had significantly more binding sites for both $PGE_2$ and $PGF_{2\alpha}$ than did SLC. However, it should be noted that no adequate control for the superovulatory treatment was carried out and therefore an effect of hCG on CL development and subsequent biochemical and functional properties of the two cell populations could not be ruled out. In addition, although the SLC fraction was devoid of LLC, the LLC fraction typically contained from 20 to 50 percent small cells. Harrison and colleagues (1987) reported no difference in the numbers of receptors for LH on LLC and SLC on days 10 and 15 of the estrous cycle in nonsuperovulated ewes and suggested that the discrepancy between their findings and those of Fitz and coworkers (1982) were the result of the PMSG treatment used in the earlier study. More recently, however, Hild-Petito and coworkers (1987) concluded that CL from superovulated ewes were functionally similar to those obtained from cyclic nonsuperovulated of ewes because the ratio of SLC to LLC and differential regulation of steroidogenesis by the two cell types were similar for both groups.

Functional differences in the regulation of steroidogenesis between large and small luteal cells have been reported for cows (Koos and Hansel, 1981; Alila *et al.*, 1988) and sheep (Fitz *et al.*, 1982). Koos and Hansel (1981) used enzymatic dispersion followed by unit gravity sedimentation to separate bovine large and small luteal cells. The resultant small cell fraction contained no LLC, however, the LLC fraction contained predominantly SLC (75%) with LLC in minority (25%). In the absence of LH, and after correction for the contribution of SLC contaminating the LLC fraction, bovine LLC were reported to produce about 20 times more progesterone in 1 hr compared with SLC. In the presence of LH (5 ng/ml), SLC progesterone production increased 11-fold in the first hour of incubation compared with the less than twofold increase for LLC. In response to a lower dose of LH (0.5 ng/ml), SLC but not LLC demonstrated increased progesterone production suggesting that SLC are more responsive to LH than are LLC. Further, addition of $PGF_{2\alpha}$ (100 ng/ml) either alone or in combination with LH (5 ng/ml) failed to significantly alter progesterone production by either large or small cells. These data suggested that small and large luteal cells are functionally different with respect to the regulation of progesterone synthesis *in vitro*. Large luteal cells demonstrate greater basal secretion of progesterone but are relatively unaffected by LH treatment whereas small luteal cells have a lower basal secretion of the steroid but respond strongly to LH stimulation. Similar results have been reported for basal and LH-induced progesterone production by ovine large and small luteal cells *in vitro* (Fitz *et al.*, 1982). However, further investigation of the differential production of progesterone by ovine luteal cells has suggested that basal secretion of progesterone may be greater in LLC because these cells have a greater quantity of mitochondria compared with SLC (Kenny *et al.*, 1989). In addition it has been proposed that the inability of ovine LLC

to respond to cAMP with increased progesterone secretion may be due to the unavailability of specific phosphoprotein substrates for protein kinase A (PKA) because fewer endogenous proteins (3 vs 7) acted as substrate for endogenous PKA in the cytosol fraction of large compared with small luteal cells, respectively (Hoyer and Kong, 1989).

Because isolation of relatively pure populations of large (90 to 99% purity) and small (100% purity) luteal cells was possible using unit gravity sedimentation followed by flow cytometry, the effects of LH and $PGF_{2\alpha}$ on these two cell types from bovine CL were reexamined (Alila *et al.*, 1988). Only extremely high concentrations of LH (100 ng/ml or greater) significantly increased progesterone production in LLC whereas progesterone production was increased sixfold in SLC treated with as little as 1 ng/ml LH. In contrast to previous results (Koos and Hansel, 1981), treatment of small luteal cells with $PGF_{2\alpha}$ resulted in a dose dependent increase in progesterone secretion and addition of $PGF_{2\alpha}$ in combination with LH resulted in further synthesis of the steroid. Basal progesterone secretion in LLC was unaffected by addition of $PGF_{2\alpha}$ alone (1000 ng/ml), similar to previous reports. Treatment of LLC with $PGF_{2\alpha}$ and LH resulted in a 50 percent reduction in progesterone synthesis. A differential response to phorbol ester (an activator of protein kinase C; PKC) treatment was also observed between the two cell types. Progesterone production was significantly increased in SLC treated with a phorbol ester (50 nM; 4$\beta$-phorbol 12, 13-dibutyrate), however, phorbol ester (20 or 100 nM) failed to alter progesterone synthesis in LLC.

A differential response to phorbol ester (phorbol 12-myristate, 13-acetate; PMA) by large and small luteal cells from CL of superovulated ewes has also been reported (Hoyer and Marion, 1989). Treatment of ovine small luteal cells *in vitro* with PMA (0.1 nM to 10

$\mu$M) failed to significantly increase progesterone secretion over that for controls. Similar incubation of an enriched population of LLC (10 to 30% contamination with small cells) with PMA resulted in significant attenuation of basal progesterone secretion at 100 nM and 10 $\mu$M concentrations. Cell viability was not altered by PMA treatment. Collectively, data from these experiments suggest that progesterone synthesis may be differentially regulated in small and large luteal cells of the cow and ewe and presumably these differences play a role in providing the proper endocrine environment for the establishment and maintenance of pregnancy.

Although the cAMP response system is generally accepted as the primary effector system through which LH exerts its effects on luteal tissue (Marsh, 1976), activation of the $Ca^{2+}$-protein kinase C (PKC) second messenger system by phorbol ester can stimulate progesterone secretion in bovine SLC and ovine LLC, however, the biological significance of this finding has yet to be determined. The conflicting results, between cell types and species, obtained after treatment of luteal cells with phorbol ester may be explained by variable expression of specific isoforms of PKC (for review, see Nishizuka, 1989; Parker *et al.*, 1989; Stabel and Parker, 1991), that have different binding affinity for phorbol ester, as has been suggested by Davis (1991). Interestingly, PMA and a calcium ionophore (A23187) have been used to mimic the luteolytic affect of $PGF_{2\alpha}$ in isolated rat luteal cells (Baum and Rosberg, 1987), leading these authors to suggest that regulation of PKC may be involved in luteal regression in this species. The negative response of ovine LLC to PMA treatment in addition to the observation that LLC contain the majority of luteal $PGF_{2\alpha}$ receptors lends support to this premise.

***Regulation by LH***

Luteinizing hormone is generally accepted to be the primary luteotropic hormone in cows and ewes and is essential for the maintenance of the CL during the luteal phase of the estrous cycle. Mason and Savard (1964) were the first to demonstrate that LH could stimulate progesterone secretion from luteal tissue *in vitro*, thus establishing the luteotropic nature of this gonadotropin in the regulation of bovine luteal function. Similar results were subsequently reported for ovine luteal tissue *in vitro* (Kaltenbach *et al.*, 1967). *In vivo* experiments using both cows and sheep confirmed the luteotropic action of LH on the CL. Administration of LH to pituitary intact cycling heifers prolonged the life span of the corpus luteum (Donaldson and Hansel, 1965b), whereas daily administration of LH antiserum to intact or hysterectomized heifers from days 2 to 6 of the cycle decreased luteal weight and progesterone content (Snook *et al.*, 1969). Similarly, infusions of LH into hypophysectomized cycling or pregnant ewes maintained luteal function (Kaltenbach *et al.*, 1968; Karsch *et al.*, 1971) and stimulated secretion of progesterone (Domański *et al.*, 1967). Administration of LH antiserum to cycling ewes resulted in luteal regression (Fuller and Hansel, 1970). Collectively, these data demonstrated that the luteotropic effects of LH were common to both ewes and cows.

LH Receptor

Luteinizing hormone exerts its effects on the CL through a specific receptor that resides in the plasma membrane of luteal cells. The structural and functional aspects of gonadotropins (Sairam, 1989; Gharib *et al.*, 1990) and the molecular structure and characteristics of the LH/CG receptor (Ascoli and Segaloff, 1989; Rajaniemi *et al.*, 1989; Leers-Sucheta and Stormshak, 1991; Segaloff, 1991) have been recently reviewed. Briefly,

LH is a member of a group of related glycoprotein hormones that includes FSH, thyroid stimulating hormone (TSH) and hCG. These glycoproteins are heterodimeric structures that share, within species, a common $\alpha$-subunit that is noncovalently associated with a distinct $\beta$ subunit, that confers hormonal specificity. Because the $\beta$-subunits of LH and hCG are nearly identical both of these hormones can bind to the same receptor, now commonly referred to as the LH/CG receptor. The presence of internal disulfide bridges within each subunit of the hormone molecule stabilizes its tertiary structure. Both $\alpha$- and $\beta$-subunits contain N-linked oligosaccharides, however, the amount and type of glycosylation varies among hormones.

The structure of the LH/CG receptor had been surrounded by controversy as some researchers reported that the receptor was composed of a single subunit while others favored a multiple subunit structure (for references see Ascoli and Segaloff, 1989). This controversy has been resolved with the isolation of the complementary DNA (cDNA) for the rat luteal (McFarland *et al.*, 1989) and porcine testis (Loosfelt *et al.*, 1989) LH/CG receptor. The mature, unglycosylated rat luteal LH/CG receptor is composed of 674 amino acids and is estimated to be 75 kDa. The N-terminal end is hydrophilic and constitutes the putative extracellular domain of the receptor and is characterized by six potential sites for N-linked glycosylation. The carboxy terminus of the receptor molecule resides within the cell and shares sequence homology with other members of the guanine nucleotide regulatory protein (G protein) -coupled receptor family. This domain contains seven putative transmembrane regions, a feature common to bovine rhodopsin, substance K, $\beta_2$-adrenergic and serotonin receptors, and also includes several potential phosphorylation sites as well as two potential proteolytic cleavage sites that may be targets for cellular control of receptor function. The

site at which the G protein is presumed to couple with the receptor has been linked to a conserved sequence of six or seven amino acid residues at the carboxy-terminal end of the third cytoplasmic loop. The LH/CG receptor cloned from porcine testis has the same overall structure and shares 89 percent amino acid identity with the receptor found in rat luteal tissue. The cDNAs for rat Sertoli FSH (Sprengel *et al.*, 1990) as well as human (Nagayami *et al.*, 1989) and canine (Parmentier *et al.*, 1989) TSH receptors have also been cloned and appear to share significant homology with the LH/CG receptor. Recently, the structural organization of the rat LH receptor gene and proposed functions of the exons has been reported (Ji and Ji, 1991; Tsai-Morris *et al.*, 1991).

Specificity of action of the glycoprotein hormones is conferred by the $\beta$-subunit and it appears that activation of the target cell requires the presence of the $\alpha\beta$ heterodimer because isolated subunits alone provoke little or no response. Glycosylation is also required for activation of adenylate cyclase (AC), however, the deglycosylated hormone is able to bind to the receptor and in some instances with greater affinity than the native hormone (Sairam, 1989). Binding of hormone to and subsequent activation of the LH receptor is a complex event. Although both subunits have been demonstrated to interact with the receptor (Pierce and Parsons, 1981), Milius and coworkers (1983) have proposed that the binding of hCG to its receptor initially involves a specific low-affinity interaction of the $\beta$-subunit with the receptor which in turn activates an additional site for the high affinity binding of the $\alpha$-subunit and concomitant stabilization of the hormone-receptor complex. This concept is supported by kinetic (Katikineni *et al.*, 1980) and hormone-receptor cross-linking (Petäjä-Repo and Rajaniemi, 1990) data.

G Proteins

Luteinizing hormone provokes progesterone synthesis in luteal cells by binding to specific high affinity LH/CG receptors localized in the plasma membrane. Transmission of the hormone signal from the hormone-receptor complex to the interior of the cell occurs though the activation of a G protein which in turn activates AC, catalyzing the conversion of ATP to cAMP. The hormone signal is then propagated by the second messenger cAMP, which activates cAMP-dependent protein kinases that phosphorylate specific proteins that activate progesterone biosynthesis (Hunzicker-Dunn and Birnbaumer, 1985).

The role of various G proteins in signal transduction has been extensively reviewed (Neer and Clapham, 1988; Weiss *et al.*, 1988; Freissmuth *et al.*, 1989; Birnbaumer, 1990; Birnbaumer *et al.*, 1990). Hormone stimulated AC activity is directly regulated by two distinct G proteins designated $G_s$ (stimulatory) and $G_i$ (inhibitory) for their action on this enzyme. Both $G_s$ and $G_i$ are heterotrimeric, composed of three subunits designated $\alpha$, $\beta$ and $\gamma$ in order of decreasing mass. Different G proteins are distinguished by unique $\alpha$-subunits which contain the nucleotide binding site. The $\beta$ and $\gamma$ subunits are found in close association ($\beta\gamma$ dimer) and are presumed to be shared among all G proteins. Cholera and pertussis toxin have been used to classify various G proteins (not just $G_s$ and $G_i$) based on their ability to ADP-ribosylate the $\alpha$-subunit. Although both bacterial toxins catalyze the transfer of ADP from NAD to specific amino acid residues on the $\alpha$-subunit, cholera toxin acts on $G_s$ whereas pertussis toxin prefers $G_i$ and the effects of $\alpha$-subunit ribosylation, on AC activity, differ between the two G proteins. Treatment of $G_s$ with cholera toxin results in persistent activation of AC whereas treatment of $G_i$ with pertussis toxin uncouples the

receptor from the G protein and releases AC from negative control by the hormone-receptor complex.

Receptor-mediated activation of G proteins has been well described by Birnbaumer (1990). The binding of LH to its receptor on luteal cells stimulates AC activity through a complex series of events in which the G protein becomes activated. Under basal conditions $G_s$ exists as a heterotrimer in which GDP is bound with high affinity to the $\alpha$-subunit ($G_{s\alpha}$). Association of the heterotrimer with the LH receptor is provoked by high affinity binding of LH to the receptor, which in turn stimulates GDP release from and GTP binding to $G_{s\alpha}$ resulting in activation of the G protein ($G^{*GTP}$). This activation step is both $Mg^{2+}$ and GTP dependent. The activated G protein, $G^{*GTP}$, is stabilized by dissociation of the $\beta\gamma$ dimer and leaves the activated $\alpha^{*GTP}$ associated with the hormone-receptor complex (H-R). Dissociation of H-R from $\alpha^{*GTP}$ allows H-R to interact with other $G_s^{GDP}$ molecules, thereby initiating a new G protein activation cycle and subsequent amplification of the LH signal. Free from the H-R complex, $\alpha^{*GTP}$ exerts its stimulatory effects on AC to increase production of cAMP from ATP. Because $G_\alpha$ contains intrinsic GTPase activity, $\alpha^{*GTP}$ is hydrolyzed to $\alpha^{GDP}$, which inactivates the G protein and frees AC to interact with another $\alpha^{*GTP}$molecule. Inactive $\alpha^{GDP}$ reassociates with the $\beta\gamma$ dimer to regenerate the holo-G protein ($G_s^{GDP}$), which may then associate with another H-R complex.

Adenylyl Cyclase and PKA

The role of adenylyl cyclase in signal transduction has been well documented in the literature (for review, see Krupinski, 1991; Levitzki and Bar-Sinai, 1991), however, because of the unstable nature of this enzyme, purification was difficult and resulted in a paucity of information on its structure and characteristics. Purification of AC from rabbit myocardial

and rat brain (Pfeuffer and Metzger, 1982) as well as bovine brain (Smigel, 1986) was achieved using affinity column chromatography. Analysis of purified bovine brain AC using SDS-PAGE, revealed a single polypeptide with an apparent $M_r$ of 120,000 daltons. More recently, the molecular characteristics of AC have been determined using cDNAs isolated from bovine brain (Krupinski *et al.*, 1989). Expression of a putative full-length cDNA from three clones in transfected COS-m6 cells resulted in enhanced AC activity that was dependent upon the amount of plasmid transfected and concentration of forskolin (a potent activator of AC) added, suggesting that the cDNA did in fact encode AC. Hydropathy analysis of the protein sequence deduced from the cDNA revealed two hydrophobic regions each containing six putative membrane-spanning helices. The amino terminus of the protein contains a six amino acid sequence that is identical to a highly conserved region of the guanine nucleotide binding pocket of G proteins. Because the proposed structure for AC was similar to proposed structures for various ion channels these authors hinted at a potential transport role for the enzyme.

It is generally accepted that cAMP exerts its actions on luteal and other target cells through the activation of cAMP-dependent protein kinases (protein kinase A; PKA) which presumably phosphorylate key regulatory enzymes controlling specific cellular functions (for reviews, see Taylor, 1989; Taylor *et al.*, 1990). Protein kinase A is found in virtually all cell types and is inactive in the absence of cAMP. The intact holoenzyme is composed of two catalytic subunits (C) noncovalently bound to a dimeric regulatory (R) subunit containing two cAMP binding sites per subunit. The holoenzyme is induced to dissociate upon the binding of cAMP to R which frees the activated C subunits to phosphorylate cellular proteins. There are two classes of PKA, Type I and Type II, that exhibit distinct differences

in the biochemical properties of their respective RI and RII subunits. Both Type I and Type II forms of PKA have been identified in rabbit, rat and bovine luteal tissue (for references, see Hunzicker-Dunn and Birnbaumer, 1985). In addition, several isozymes of PKA containing antigenically distinct RI and RII subunits have been detected in luteal tissue of several species, including rats (Hunzicker-Dunn *et al.*, 1991) and pigs (DeManno and Hunzicker-Dunn, 1991), however, the biological significance of these PKA isoforms in the regulation of acute or chronic synthesis of progesterone remains to be determined.

Phosphoinositides and PKC

In addition to the cAMP second messenger system, it is clear that many hormones and neurotransmitters evoke their response through activation of the phosphoinositide cascade and mobilization of intra- and extra-cellular $Ca^{2+}$. Binding of the hormone or neurotransmitter to its receptor stimulates phospholipase C (presumably via a G protein) which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) in the plasma membrane of the target cell (for review, see Berridge, 1987; Exton *et al.*, 1991). Hydrolysis of this phosphoinositide releases two second messengers, inositol trisphosphate ($IP_3$) and diacylglycerol (DAG), that act indirectly and directly, respectively, to stimulate a calcium and phospholipid-dependent protein kinase (protein kinase C; PKC). Diacylglycerol binds directly to PKC and increases the affinity of the kinase for $Ca^{2+}$ whereas $IP_3$ stimulates the release of intracellular $Ca^{2+}$ stores, thus permitting activation of the kinase and subsequent phosphorylation of specific regulatory proteins at concentrations of $Ca^{2+}$ encountered within the cell.

Recently, some investigators have suggested that calcium-dependent second messengers may also mediate gonadotropin-induced progesterone synthesis in bovine (Davis

*et al.*, 1981; Hansel and Dowd, 1986) but not ovine (Wiltbank *et al.*, 1989) or rat (LaHav *et al.*, 1988) luteal tissue via phosphoinositide hydrolysis and subsequent activation of PKC. However, others have reported that LH (25-100 ng/ml) failed to stimulate phospholipid metabolism in bovine CL (Scott *et al.*, 1968). The cAMP and inositol phospholipid signalling systems in bovine luteal cells have been recently reviewed (Davis, 1991; Hansel *et al.*, 1991).

Results of some *in vitro* experiments have demonstrated that supraphysiological doses of LH (1 $\mu$g/ml) stimulated steroidogenesis in bovine luteal cells through activation of the phosphatidylinositol (PI) cascade (Davis *et al.*, 1981), an increase in free intracellular $Ca^{2+}$ (Davis *et al.*, 1987) and subsequent stimulation of PKC (Davis and Clark, 1983). More recently, Alila and coworkers (1989) evaluated the effect of LH on intracellular free $Ca^{2+}$ concentration in highly purified small and large bovine luteal cell populations. These researchers reported that resting $Ca^{2+}$ level was significantly higher in large compared with small luteal cells. Addition of LH (1 $\mu$g/ml) to small luteal cells resulted in a rapid transient rise in $Ca^{2+}$ (two to sixfold increase), due to mobilization of intracellular calcium stores, that was followed by a sustained secondary elevation due to $Ca^{2+}$ influx from extracellular sources. Addition of a more physiological dose of LH (10 ng/ml) resulted in a significant increase in $Ca^{2+}$, however, it was difficult to distinguish the two phases of the $Ca^{2+}$ response and the magnitude of response appeared to be much smaller than that observed with the higher concentration of LH. The response of large luteal cells to LH (1 $\mu$g/ml) differed from that observed in small cells in that only a single phase (1.5 to twofold increase) of LH-induced $Ca^{2+}$ response was observed and this effect was attributed entirely to the influx of extracellular $Ca^{2+}$ stores. These data demonstrate that physiological levels of LH (10 ng/ml)

can evoke a small but significant increase, and supraphysiological doses a large increase, in intracellular $Ca^{2+}$ concentration in small but not large luteal cells. The effect of a small increase in intracellular calcium on phosphoinositide hydrolysis and PKC activity in small luteal cells was not determined in these experiments and therefore these data should be viewed with some caution. In addition, previous experiments with isolated bovine luteal cell cultures have failed to demonstrate significant increases in $IP_3$ formation at physiological levels of gonadotropin (10 ng/ml) even though that dosage resulted in a small, but significant, increase in intracellular $Ca^{2+}$ levels (Davis *et al.*, 1987).

Hoyer and Kong (1989) investigated the activities and endogenous substrates of PKA and PKC in soluble fractions of ovine small and large luteal cell homogenates. The rate of incorporation of phosphate into histone IIA as stimulated by cAMP (6 $\mu$M) increased from 1 to 60 min and did not differ significantly between the two cell types. Under conditions of maximal stimulation, increased phosphotransferase activity of PKA (6.9-fold) and PKC (2.9-fold) was observed in small cells, however, only PKA activity was significantly increased (4.9-fold) in similarly treated large cells. Endogenous proteins of varying $M_r$ were observed to act as substrates for PKA and PKC in both cell types, however, the specific proteins acting as substrate for the two kinases differed between small and large cells leading these researchers to speculate that the differential regulation of progesterone biosynthesis between the two cell types may be due to the observed differences in PKC activity and endogenous proteins phosphorylated.

Several recent reports in the literature provide strong evidence that receptors for glycoprotein hormones, such as the human TSH (VanSande *et al.*, 1990) and murine LH (Gudermann *et al.*, 1992) receptors, may be functionally coupled to both the adenylyl

cyclase-cAMP and phospholipase C-phosphoinositide signal transduction pathways. The murine LH receptor was cloned and expressed in Ltk$^-$ cells (Gudermann *et al.*, 1992). This cell line was chosen because their endogenous AC and phospholipase C-phosphoinositide systems are not responsive to LH or hCG. Transfection of the cells with the cloned receptor cDNA resulted in the appearance of an LH/CG responsive AC system and the new cell line (LHR 11/6) exhibited saturable specific binding of hCG. It was subsequently determined that LHR 11/6 cells express ~20,000 LH receptor sites/cell. Addition of hCG to LHR 11/6 cells stimulated inositol phosphate production in a dose-dependent manner. Mobilization of intracellular $Ca^{2+}$ was induced upon administration of hCG (1 $\mu$g/ml) and this response was demonstrated to be specific for the LH receptor and not the result of increased cAMP levels. Clearly, these data support the concept that the LH/CG receptor is capable of coupling to two second messenger systems, however, the physiological importance or necessity for an additional signal transduction system, that is only activated by extremely high levels of LH, in luteal cells is puzzling. Gudermann and coworkers (1992) pointed out that in some instances peripheral levels of LH or hCG might be high enough to stimulate phospholipid hydrolysis and mobilization of $Ca^{2+}$, for example, during ovulation in the rat and during pregnancy in humans. In the cow, however, peak LH concentration during the preovulatory surge is generally less than 50 ng/ml (Arije *et al.*, 1974; Chenault *et al.*, 1975; Rahe *et al.*, 1980) and during pregnancy does not exceed 5 ng/ml (Arije *et al.*, 1974) suggesting that this "phenomenon" may not be of biological importance in this species.

Occupied and Unoccupied Receptors

The importance of the LH/CG receptor in the development of the CL and subsequent regulation of luteal function has been demonstrated in the preceding pages. Because target

cell sensitivity to a specific hormone has been associated with changes in the numbers of receptors for that hormone it seems reasonable to expect that progesterone biosynthesis might be regulated, in part, through alteration of luteal cell receptor numbers. Progesterone synthesis at various times during the estrous or menstrual cycle has been highly correlated to changes in the numbers of unoccupied and(or) occupied LH receptors in a number of different species, including the cow, ewe and monkey (for references, see Leers-Sucheta and Stormshak, 1990). Diekman and coworkers (1978) performed a comprehensive study that examined the relationship between the total number of LH receptors, receptors occupied by endogenous hormone, luteal weight and progesterone secretion during the estrous cycle and early pregnancy in the ewe. These researchers reported that there was a 40-fold increase in the total number of LH receptors between days 2 and 10 of the cycle at which time circulating concentrations of LH decreased. Occupied receptors increased sixfold during this period, however, this comprised only 0.6% of the total number of LH receptors. Concomitant with the increase in total and occupied receptors, CL weight and serum progesterone increased sixfold and tenfold, respectively. The affinity of LH for its receptor did not change during the cycle. Similarly, in CL of cows, total number of unoccupied LH receptors increased from days 4 to 10 of the estrous cycle and total number of occupied LH receptors per CL increased fourfold from days 4 to 10 (Garverick *et al.*, 1985). Occupied receptor concentration comprised only 2% of total receptor number and, in contrast to the ewe (Diekman *et al.*, 1978), the affinity of LH for its receptor in bovine luteal membranes showed a small but significant increase over the cycle that was not correlated to progesterone secretion (Garverick *et al.*, 1985). Although the total number of receptors and number of occupied receptors in the ewe and unoccupied receptors in the cow were highly correlated

to serum progesterone levels, the physiological significance of this finding is unclear. Because very few receptors need to be occupied to in order to maintain basal luteal function, changes in the total number of receptors, and perhaps the number of occupied receptors, may not be indicative of altered luteal cell function. Low occupancy of luteal LH receptors has also been described during the estrous cycle of the sow (0.24 to 1.02%; Ziecik *et al.*, 1980) and collectively these data support the concept of "spare" receptors, in which receptors need not be completely saturated with ligand in order to provoke a cellular response.

Steroidogenic Enzymes

Control of steroidogenesis in luteal and other tissues must rely on the regulation of the concentration and(or) activity of specific biosynthetic enzymes and availability of substrate. Production of steroidogenic enzymes and other proteins critical to steroidogenesis may be regulated at the level of gene expression or perhaps mRNA stability. Luteal progesterone synthesis requires cholesterol, which may be provided to the cells by low density lipoproteins (LDL) from the circulation, released from intracellular cholesterol-ester stores or synthesized *de novo* from acetate (for references, see Grummer and Carroll, 1988).

Rodgers and coworkers (1987a) used immunoblotting techniques to determine luteal content and regulation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a rate limiting enzyme in cholesterol biosynthesis (HMG-CoA → mevalonate), at various stages of the luteal phase (as determined by visual inspection of CL) of the bovine estrous cycle. Levels of HMG-CoA reductase were increased in CL during the mid-luteal phase as compared with CL from the early or late stages of the luteal phase. Treatment of luteal cells in primary monolayer culture with dibutyryl-cAMP (db-cAMP; 1 mM) stimulated synthesis

of the reductase, however, synthesis was not always induced by treatment with LH. Synthesis of HMG-CoA reductase was inhibited by bovine LDL and high density lipoproteins (HDL) as well as 22R-cholesterol and 25-hydroxycholesterol. The stimulatory action of db-cAMP on the synthesis of HMG-CoA reductase was attenuated by HDL and an inhibitor of cytochrome P-450 side-chain cleavage ($P\text{-}450_{scc}$; cholesterol → pregnenolone), clotrimazole (3.5 $\mu$M), and led these investigators to suggest that the action of db-cAMP was mediated primarily through changes in intracellular cholesterol content. Luteal LDL receptor mRNA, as determined by Northern blot analysis, is present during the early and midluteal phase of the cycle but not during CL regression (Rodgers *et al.*, 1987b). Although the effects of LH or cAMP on LDL mRNA synthesis were not determined in this latter study, induction of LDL and HDL receptors has been reported to be regulated by gonadotropin in rat CL (Hwang and Menon, 1983). Collectively, these data demonstrate that HMG-CoA reductase content is increased and LDL receptor mRNA is present in bovine CL during the midluteal phase of the estrous cycle when progesterone production is maximal and suggests that progesterone biosynthesis in luteal cells at this time is dependent upon cholesterol derived from *de novo* synthesis as well as cellular uptake in the form of LDL.

Several recent experiments have demonstrated that the levels of several key enzymes in the steroidogenic pathway, $P\text{-}450_{scc}$ and 3$\beta$-hydroxysteroid dehydrogenase (3$\beta$-HSD; pregnenolone → progesterone) and their mRNAs are regulated in a coordinate fashion during luteal development. Content of $P450_{scc}$ in bovine luteal cells was increased 12-fold by the early to mid-luteal phase of the estrous cycle and then decreased in regressing CL (Rodgers *et al.*, 1986) and comparable changes have been observed in $P\text{-}450_{scc}$ mRNA levels during luteal development (Rodgers *et al.*, 1987b). Luteinizing hormone-induced increase in

progesterone synthesis by bovine luteal cells in primary culture was associated with increased levels of mRNA for P-450$_{scc}$ (Stirling *et al.*, 1990) suggesting that LH either increased P-450$_{scc}$ gene (CYP11A) expression or mRNA stability. More recently, treatment of bovine luteal cells in primary culture with forskolin (25 $\mu$M) significantly elevated P-450$_{scc}$ mRNA levels suggesting that the stimulatory effect of LH on P-450$_{scc}$ gene transcription was at least partially mediated by cAMP (Lauber *et al.*, 1991). In contrast, P-450$_{scc}$ gene expression in rat luteal cells appears to be regulated via a cAMP-independent mechanism (Oonk *et al.*, 1989) suggesting that there may be species specific regulation of genes encoding steroidogenic enzymes. Lauber and coworkers (1991) investigated the possibility that the 5′-regulatory region of the CYP11A gene contained a cAMP response element (CRE) by incubating bovine luteal cells transfected with various chimeric DNA constructs in the presence and absence of forskolin (25 $\mu$M). The cloned DNA constructs contained increasing deletions of the 5′-regulatory regions of bovine CPY11A upstream of two different reporter genes, chloramphenicol acetyl-transferase (CAT) or rabbit $\beta$-globin (OVEC). The P-450$_{scc}$-CAT reporter gene contained the P-450$_{scc}$ promoter whereas the P-450$_{scc}$-OVEC gene contained the rabbit $\beta$-globin promoter. It appears that both basal (absence of forskolin) and cAMP-dependent regulation of CYP11A gene expression is controlled by a cis-acting element, presumably a CRE and perhaps a basal enhancer, located at -186 to -101 base pairs upstream from the transcriptional start site because deletion of this region drastically reduced both basal and forskolin stimulated CAT activity. In addition, basal expression of the gene appeared to require the presence of homologous promoter (P-450$_{scc}$ promoter) because basal expression of the P450$_{scc}$ -OVEC gene, but not the -CAT gene, was negligible. However, it should be noted that the specific DNA sequence of the

putative CRE (-186 to -101bp) does not share sequence homology with that of the classical CRE (Ahlgren *et al.*, 1990). Because a classical CRE (**TGACGTCA**) is lacking in the upstream regulatory regions of other P-450 genes and these genes do not share a common CRE, Simpson and colleagues (1991) have suggested that P-450 genes may interact with different members of the cAMP response element binding protein (CREB) family of transcription regulating factors.

It appears that the typical profile of progesterone secretion observed during the estrous cycle of the cow may be attributed in part to the differential regulation of yet another key enzyme in progesterone biosynthesis. Couët and coworkers (1990) detected changes in the levels of 3$\beta$-HSD mRNA, protein and activity in bovine CL during the estrous cycle, suggesting that this enzyme may not be constitutively expressed in ovarian tissue as had been previously suggested (Erickson *et al.*, 1985). Corpora lutea were assigned to specific intervals of days of the cycle based upon visual and histological analysis. Levels of 3$\beta$-HSD mRNA increased from days 1 to 3, were maximal on days 10 to 11, decreased progressively from days 12 to 17 and fell abruptly to 5% of maximal by days 18 to 20 of the estrous cycle. Immunoreactive levels and enzymatic activity of 3$\beta$-HSD paralleled the observed changes in mRNA. Immunocytochemical analysis of CL sections revealed that the enzyme was localized to the cytoplasm of both small and large cells. Because mRNA levels, protein content and activity of 3$\beta$-HSD closely paralleled one another over the duration of the estrous cycle, these researchers suggested that progesterone synthesis in the bovine CL is regulated, in part, at the level of 3$\beta$ -HSD gene expression and/or mRNA stability.

Clearly, in the cow, the coordinate expression and activity of P-450$_{scc}$ and 3$\beta$-HSD, as well as other enzymes and proteins involved in cholesterol synthesis and uptake, differ

depending on the stage of luteal development and result in the distinct profile of progesterone secretion observed during the luteal phase of the estrous cycle. The regulatory mechanism(s) underlying the expression and activity of these enzymes is presumed to be mediated by LH-induced production of cAMP, however, it must be remembered that only small luteal cells are responsive to LH and cAMP (Hoyer *et al.*, 1984) and that most of the progesterone secreted during the luteal phase of the cycle is produced by the large cells (Niswender *et al.*, 1985). Therefore, regulation of steroidogenesis in large luteal cells may occur through a yet to be discovered cAMP-independent mechanism or as suggested by Niswender and Nett (1988) large luteal cells may be deficient in the ability to reduce cAMP levels or cAMP-dependent processes. It would be of great interest to determine if the expression of the genes or stability of mRNA for regulatory enzymes in cholesterol and progesterone biosynthesis and LDL receptor differs between small and large cells during the estrous cycle, however, this may be a difficult task as small luteal cells significantly outnumber large luteal cells during the cycle and large cells appear to be more sensitive to separation techniques. Clearly, further research is required to determine the underlying mechanism that controls progesterone biosynthesis in the large luteal cell.

***Secretion of LH During the Luteal Phase***

The secretory pattern of LH during the luteal phase of the cycle (tonic LH secretion) has been reported for many species including cows (Rahe *et al.*, 1980) and ewes (Yuthasastrakosol *et al.*, 1977). In the cow during the early luteal phase (day 3) LH pulses were classified as low amplitude (ΔLH, 0.3-1.8 ng) and high frequency (20 to 30 pulses/24 hr). Midluteal (days 10 to 11) pulses of LH were classified as high amplitude (ΔLH, 1.2-7.0 ng) and low frequency (6-8 pulses/ 24 hr) and this pattern appeared to be maintained

until late in the cycle, several days prior to ovulation (Rahe *et al.*, 1980). The observed changes in the amplitude and frequency of LH release were suggested to be due changes in the ovarian steroid milieu that occur over the course of the estrous cycle.

Estradiol and progesterone have been demonstrated to exert differential effects on the tonic secretion of LH (Goodman and Karsch, 1980). Treatment of OVX ewes with steroid implants, to produce luteal levels of estradiol (2-3 pg/ml) or progesterone (3-5 ng/ml), partially suppressed LH secretion, however, the inhibitory effect was manifested differently by the two steroids. Estradiol decreased pulse amplitude with no effect on pulse frequency whereas progesterone decreased the frequency but did not affect the amplitude of the pulses. A differential effect on LH secretion was also observed in response to GnRH (8 ng/kg body weight). Estradiol, but not progesterone, markedly decreased the height of the GnRH-induced LH peak. Collectively, these data led Goodman and Karsch (1980) to suggest that progesterone suppresses LH pulse frequency by acting at the hypothalamus to reduce the frequency of GnRH pulses whereas estradiol may act to suppress pituitary responsiveness to LH, resulting in reduced LH pulse amplitude.

Because low levels of progesterone (1 ng/ml) have been reported to suppress LH only in the presence of estradiol (Goodman *et al.*, 1980) it was suggested that estradiol and progesterone act synergistically to suppress tonic LH secretion during the luteal phase of the estrous cycle in the ewe. The mechanism whereby estradiol facilitates the negative effects of progesterone on tonic LH secretion was investigated in estradiol-treated OVX ewes administered low levels of progesterone (Goodman *et al.*, 1981). In the absence of estradiol, luteal levels, but not low levels, of circulating progesterone reduced LH pulse frequency and mean serum LH concentration. In contrast, low levels of progesterone markedly reduced

LH pulse frequency and decreased mean LH concentrations in estradiol-treated ewes. Because progesterone, in the presence of estradiol, reduced LH pulse frequency without altering pulse amplitude these authors proposed that estradiol may increase the sensitivity of the hypothalamus to progesterone negative feedback, resulting in reduced LH pulse frequency.

Although, estradiol has been demonstrated to regulate expression of the genes encoding the $\alpha$- and $\beta$- subunits of LH in the ewe (Nilson *et al.*, 1983) it appears that progesterone alone (in the absence of estradiol) does not exert its negative feedback effects on tonic LH secretion through alteration of gonadotropin gene expression, pituitary content of LH or GnRH receptor numbers (Hamernik *et al.*, 1987). However, recently, Girmus and Wise (1991) have proposed that progesterone directly inhibits pituitary LH release in ewes exposed simultaneously to estradiol and progesterone. In this study OVX- hypothalamic-pituitary-disconnected (OVX-HPD) ewes were implanted with silastic capsules containing progesterone and/or estradiol after 7 days of exogenous GnRH infusion (400 ng/3 min pulse every 2 hr). Blood samples were collected on day 7, prior to implant insertion, and on day 14 of GnRH infusion, after implants had been in place for 1 wk. Following the last sampling period on day 14, pituitaries were collected for subsequent determination of LH subunit mRNA concentrations and LH content. Administration of either steroid alone failed to alter LH pulse amplitude whereas simultaneous implantation of progesterone and estrogen resulted in a 70 percent reduction in pulse amplitude. Relative $\alpha$-subunit mRNA concentrations were not affected by either steroid alone or the combination treatment. Estradiol, either alone or in the presence of progesterone, significantly reduced LH-$\beta$ subunit concentrations as compared with GnRH-infused control ewes. Progesterone alone had no

effect on LH-$\beta$ subunit mRNA levels. Estradiol alone significantly reduced pituitary content of LH whereas progesterone alone or in combination with estradiol had no effect on LH content. Because simultaneous administration of estrogen and progesterone, but not either steroid alone, reduced LH pulse amplitude in the face of a constant frequency and amplitude of GnRH release, these authors suggested that steroidal regulation of tonic LH secretion may be exerted, in part, through direct inhibition of pulsatile LH release by the synergistic action of progesterone and estradiol. Further, direct inhibition of LH release by the two steroids together was not the result of reduced LH synthesis because the reduction in LH-$\beta$ subunit mRNA observed with the combination treatment could be accounted for by the effect of estradiol alone. Similarly, the inhibition was not the result of reduced pituitary stores of LH because treatment with both steroids failed to alter pituitary content of LH even though LH pulse amplitude was decreased. From these data it appears that regulation of LH synthesis and release by progesterone and estradiol may be dissociated, however, the precise mechanism(s) underlying the synergistic inhibition of tonic LH secretion in the ewe have yet to be determined.

Clearly, estradiol and progesterone interact in a complex manner at both the hypothalamus and pituitary to regulate LH secretion during the luteal phase of the estrous cycle of the ewe and presumably the cow. These steroids appear to regulate different aspects of LH secretion, progesterone acts at the hypothalamic level to reduce LH pulse frequency whereas estradiol suppresses LH pulse amplitude at the level of the pituitary. In addition to these well accepted sites of action, estradiol may act at the hypothalamus or at extrahypothalamic sites to increase sensitivity to progesterone negative feedback, and

progesterone, in the presence of estradiol, may also act at the pituitary directly to reduce the secretion of LH.

**Regression of the Corpus Luteum**

Because the life span of the CL determines the length of the estrous or menstrual cycle, early research focused on elucidating the factor(s) and mechanism controlling the demise of this ovarian structure. It has been well established in many species that the uterus influences ovarian function (Anderson *et al.*, 1979). A number of experiments using cows (Hansel and Snook, 1970; Ginther, 1974) and ewes (McCracken *et al.*, 1971) have demonstrated that the uterus and ovary must be in close proximity for luteal regression to occur in these species. Babcock (1966) first suggested that prostaglandins might be luteolytic in cattle and it is now generally accepted that $PGF_{2\alpha}$ of uterine origin is the luteolysin in cattle, sheep, horses, swine, guinea pigs, rabbit, hamster, and pseudopregnant rats. In contrast, the uterus is not thought to be a source of endogenous luteolysin in the mouse, primate and human because hysterectomy has no effect on cyclic ovarian function in these species, however, administration of $PGF_{2\alpha}$ *in vivo* does induce luteal regression suggesting that an extrauterine source of $PGF_{2\alpha}$ may be involved in luteolysis (for review, see Horton and Poyser, 1976; Auletta and Flint, 1988; Silvia *et al.*, 1991). Recently, it has been suggested that luteolysis may occur in the monkey through the local action of an ovarian or CL-derived luteolytic factor, presumed to be $PGF_{2\alpha}$, and that luteal function may be regulated through alteration in the local production or action of stimulatory prostaglandins ($PGD_2$, $PGE_2$ and $PGI_2$) and inhibitory $PGF_{2\alpha}$ within the primate ovary (Stouffer, 1991).

***Prostaglandin $F_{2\alpha}$***

Early transplantation experiments in ewes (McCracken *et al.*, 1971) and cows (Hansel and Snook, 1970) demonstrated that physical separation of the ovary from the uterus extended the life span of the CL, whereas administration of $PGF_{2\alpha}$ resulted in premature luteal regression. In addition, endometrial and uterine venous plasma concentrations of $PGF_{2\alpha}$ in Holstein heifers were reported to be low from days 1 to 14 and were increased from day 15 to estrus (Shemesh and Hansel, 1975). Further, passive immunization of cows and ewes with $PGF_{2\alpha}$ antibodies successfully prevented luteal regression (Fairclough *et al.*, 1981). Collectively, these data support the concept that uterine $PGF_{2\alpha}$ is the endogenous luteolysin in ewes and cows.

In cows (Ginther, 1974; Hansel, 1975) and ewes (McCracken *et al.*, 1971), the mechanism of transfer of $PGF_{2\alpha}$ from the uterus to the ovary appears to be localized and one in which $PGF_{2\alpha}$ leaves the uterus *via* the uterine vein and by counter-current transfer passes through the wall of the utero-ovarian vein and into the ovarian artery. This hypothesis is supported by experiments in which the ovarian artery was physically separated from the utero-ovarian vein in the ewe (Barrett *et al.*, 1971) and by experiments involving anastomoses of the vasculature in unilaterally hysterectomized cows (Mapletoft *et al.*, 1976). In the first case, physical separation of the two vessels resulted in luteal maintenance and in the latter case, luteolysis of the CL in the ovary ipsilateral to the hysterectomized horn occurred only when the uterine vein from the remaining horn was anastomosed to the ovarian artery supplying the ovary bearing the CL. In addition, intrauterine administration of $PGF_{2\alpha}$ to cows resulted in a greater concentration of the fatty acid in ovarian arterial compared with carotid or jugular blood (Hixon and Hansel, 1974), demonstrating a

preferential transfer of $PGF_{2\alpha}$ to the ovarian artery and supporting a local rather than systemic action of the luteolysin.

The precise mechanism(s) through which $PGF_{2\alpha}$ acts to induce luteolysis is not well understood, however, two hypotheses have been proposed to explain the luteolytic action of this eicosanoid. The first hypothesis suggests that luteal regression occurs in response to $PGF_{2\alpha}$-induced reduction in blood flow to the ovary bearing the CL (Pharriss *et al.*, 1970) whereas the second involves a direct action of $PGF_{2\alpha}$ on luteal cells. Evidence supporting reduced blood flow as a luteolytic mechanism of $PGF_{2\alpha}$ action has been reported for the ewe. Serum progesterone and blood flow to the ovary bearing the CL decrease during natural (Ford *et al.*, 1979) or $PGF_{2\alpha}$-induced (Nett *et al.*, 1976) luteal regression in this species, however, it is still unclear if reduced ovarian blood flow is a result, rather than the cause, of CL demise.

There is also evidence to support a direct action of $PGF_{2\alpha}$ on luteal cell function in cows and ewes. Incubation of ovine luteal slices *in vitro* with $PGF_{2\alpha}$ attenuated LH-stimulated progesterone synthesis (Fletcher and Niswender, 1982). However, $PGF_{2\alpha}$ inhibited progesterone production by HDL-stimulated ovine large but not HDL or LH-stimulated small luteal cells (Wiltbank *et al.*, 1990) suggesting that the inhibitory effect was mediated through the large cell. In contrast, $PGF_{2\alpha}$ provoked progesterone synthesis in bovine SLC and inhibited LH or cAMP stimulated steroidogenesis in LLC similar to that reported for the ewe (Hansel *et al.*, 1991).

Although LLC contain the majority of receptors for $PGF_{2\alpha}$ and $PGE_2$ and it seems likely that the luteolytic effects of $PGF_{2\alpha}$ are mediated through this cell type, some researchers have suggested that the inhibitory effects of $PGF_{2\alpha}$ on progesterone secretion

may involve the interaction of large and small luteal cells (Fitz *et al.*, 1982). Incubation of ovine small luteal cells with $PGF_{2\alpha}$ had no effect on LH-stimulated progesterone secretion, however, when small cells were contaminated with large cells, treatment with $PGF_{2\alpha}$ significantly inhibited steroid production, suggesting that large luteal cells were required to mediate the inhibitory effect of the eicosanoid (Rodgers *et al.*, 1985).

Recently, gap junction-mediated intercellular communication between bovine luteal cells was investigated using a dye-coupling technique in conjunction with interactive laser cytometry (Redmer *et al.*, 1991). These investigators reported that small luteal cells communicated rapidly with each other and that communication was not influenced by LH (100 ng/ml) or $PGF_{2\alpha}$ (1 $\mu$M) but was increased by forskolin (1 $\mu$M) treatment. Communication between large and small luteal cells was significantly enhanced by treatment with LH or $PGF_{2\alpha}$ alone but not by a combination of the two treatments. Forskolin also stimulated large cell-small cell communication. Interestingly, large luteal cells did not communicate with each other under any of the treatment conditions tested. These data demonstrate that luteal cells are capable of intercellular communication *via* gap junctions and that the rate of communication may be hormonally regulated. In addition, these data lend support to the concept that the luteolytic effects of $PGF_{2\alpha}$ may involve intercellular communication, in which large luteal cells bind $PGF_{2\alpha}$ and propagate the signal to the small cells *via* gap junctions, as has been previously suggested (Fitz *et al.*, 1982).

Contrary to popular belief, Milvae and coworkers (1991a) have suggested that granulosa-derived (large) luteal cells have a limited role in regulating the life span of the CL. These researchers reported that removal of a large number of granulosa cells (range = 8.3 to 16.4 $\times$ $10^6$; estimated 61 to 86% of total) from the preovulatory follicle of heifers

resulted in an 80 percent decrease in progesterone on days 7 to 17 of the estrous cycle compared with control heifers and heifers in which granulosa cells had been removed and then immediately returned to the follicle. Surprisingly, removal of granulosa cells and the subsequent reduction in serum progesterone did not affect estrous cycle length, prompting these investigators to suggest that if $PGF_{2\alpha}$ is involved in luteolysis it may not be acting on granulosa-derived luteal cells. An alternative interpretation is that $PGF_{2\alpha}$ may be acting through large luteal cells to cause luteal regression, however, it may not take many large cells to convey the luteolytic signal to the small cells. In addition, although Milvae and coworkers (1991a) reported that subtracting the steroidogenic contribution of granulosa-derived large luteal cells could not, alone, account for the observed 80 percent decrease in serum progesterone, Niswender and colleagues (1985) have reported that greater than 80 percent of progesterone secreted by the ovine CL is derived from large luteal cells.

At the cellular level, the mechanism whereby $PGF_{2\alpha}$ invokes luteal regression in domestic ruminants is not well understood, however, it is presumed to act initially through membrane-bound receptors on large luteal cells, resulting in an increase in phospholipid hydrolysis (McCann and Flint, 1987; Davis *et al.*, 1988) and intracellular calcium levels (Alila *et al.*, 1989; Wegner *et al.*, 1990) and may involve activation of PKC (Hoyer and Marion, 1989; Wiltbank *et al.*, 1990). Hansel and coworkers (1991) have suggested that the large increase in intracellular $Ca^{2+}$ that occurs in bovine large luteal cells treated with both LH and $PGF_{2\alpha}$ (greater than the response induced by either treatment alone) may be cytotoxic, thereby providing a direct luteolytic role for $Ca^{2+}$ in large cells. Similarly, $PGF_{2\alpha}$ induced a transient increase in intracellular $Ca^{2+}$ levels in ovine large but not small luteal cells (Wegner *et al.*, 1990) and basal secretion of progesterone from large cells was

attenuated by a sustained decrease or increase in intracellular $Ca^{2+}$ suggesting that optimal secretion of this steroid may occur only within a specific range of intracellular $Ca^{2+}$ concentration (Wegner *et al.*, 1991). Further, these authors suggested that inhibition of progesterone synthesis by $PGF_{2\alpha}$-induced sustained elevations of $Ca^{2+}$ may result from the cytotoxic properties of this element, possibly as a result of redistribution of $Ca^{2+}$ among intracellular compartments, reduction in membrane fluidity or inhibition of cholesterol transport to mitochondria. Recently, Grusenmeyer and Pate (1992) have reported that although $PGF_{2\alpha}$ inhibited lipoprotein-stimulated progesterone production in bovine luteal cells *in vitro*, it failed to inhibit lipoprotein-induced increases in cellular or mitochondrial cholesterol content. Further, $PGF_{2\alpha}$ does not appear to directly inhibit $P450_{scc}$ because $PGF_{2\alpha}$ (10 ng/ml) failed to block 25-hydroxycholesterol-induced progesterone synthesis and prompted these investigators to suggest that $PGF_{2\alpha}$ may be acting at a site distal to cholesterol transport to the mitochondria, but prior to side-chain cleavage, to attenuate progesterone biosynthesis.

Several *in vitro* experiments have suggested that PKC may be involved in $PGF_{2\alpha}$-induced inhibition of luteal progesterone synthesis in cows and sheep. Progesterone production in HDL-stimulated ovine large luteal cells was inhibited in a dose-dependent manner by $PGF_{2\alpha}$, however, when these cells were made PKC-deficient by treatment with phorbol-12-myristate-13-acetate (PMA; 1 $\mu$M) for 12 hr, administration of $PGF_{2\alpha}$ failed to alter HDL-stimulated progesterone secretion and prompted these investigators to suggest that PKC may be involved in the antisteroidogenic effect of $PGF_{2\alpha}$ observed in large cells (Wiltbank *et al.*, 1990). *In vitro* incubation of ovine large luteal cells with PMA (100 nM) for 2 hr to stimulate PKC activity, significantly reduced progesterone secretion and

administration of sphingosine (1 to 100 $\mu$M), a specific PKC inhibitor, also suppressed progesterone production, however, sphingosine-induced inhibition of steroidogenesis was significantly correlated with decreased cell viability (Hoyer and Marion, 1989). In contrast, progesterone production by bovine large luteal cells is unaffected by PMA treatment (Alila *et al.*, 1988), however, as was mentioned previously, PKC isozymes may vary in their responsiveness to PMA and there may be species specific differences in the distribution of PKC isozymes in the CL, which could explain the different responses reported for ovine and bovine luteal cells incubated with phorbol ester.

Although it is generally accepted that $PGF_{2\alpha}$ is the endogenous luteolysin in both sheep and cattle, the exact mechanism underlying luteal demise at the end of the estrous cycle is poorly understood. It now appears that phosphoinositide hydrolysis, intracellular $Ca^{2+}$ and intramitochondrial cholesterol mobilization and PKC activation may participate in a complex manner to initiate and mediate the luteolytic signal in large luteal cells. In addition $PGF_{2\alpha}$ has been demonstrated to reduce ovarian blood flow, which may also effect luteal cell viability. There is also evidence to suggest that $PGF_{2\alpha}$ increases the rate of contact-dependent communication between large and small luteal cells and raises the possibility that the luteolytic signal is transferred from large cells to adjacent small cells *via* gap junctions. Further research is required to determine the precise role of $PGF_{2\alpha}$-stimulated effector systems, as well as the significance of hormone-responsive communication among luteal cells, in the regulation of luteal function.

***Oxytocin***

Prostaglandin $F_{2\alpha}$ has been directly measured in utero-ovarian venous plasma of sheep (McCracken *et al.*, 1972) and cattle (Nancarrow *et al.*, 1973) during luteal regression.

Analysis of the subsequent hormone profiles revealed that uterine release of the eicosanoid occurred several days prior to estrus and was pulsatile in nature. Because systemic $PGF_{2\alpha}$ is rapidly degraded in the lungs (Piper *et al.*, 1970) and sampling uterine venous drainage is difficult and inconvenient, a great deal of research has relied on determination of the primary plasma metabolite of $PGF_{2\alpha}$, 15-keto-13,14-dihydro-$PGF_{2\alpha}$ (PGFM) and the assumption that levels of this metabolite accurately reflect secretion of uterine $PGF_{2\alpha}$. It should be noted, however, that controversy surrounds the soundness of this assumption (Silvia *et al.*, 1991). The validity of using peripheral PGFM levels as an estimate of uterine $PGF_{2\alpha}$ release is supported by experiments in ewes (Zarco *et al.*, 1988) and cows (Peterson *et al.*, 1975; Kindahl *et al.*, 1976) in which the secretory profile of the metabolite at the end of the estrous cycle was similar to that previously reported for $PGF_{2\alpha}$. Therefore, in domestic ruminants, it has been demonstrated that $PGF_{2\alpha}$ and PGFM occur as discrete pulses in the circulation that appear 2 to 3 days prior to estrus when circulating concentrations of progesterone are still elevated.

It is generally accepted that the pulsatile release of uterine $PGF_{2\alpha}$ that induces luteal regression in ewes and cows is stimulated by oxytocin, a nonapeptide ($Cys^1$-$Tyr^2$-$Ile^3$-$Gln^4$-$Asn^5$-$Cys^6$-$Pro^7$-$Leu^8$-$Gly^9$-$NH_2$) containing an internal disulfide bond linking cystine residues at positions 1 and 6 (for review, see Schams, 1989; Wathes, 1989; Flint *et al.*, 1990; Silvia *et al.*, 1991). Early research clearly demonstrated that exogenous oxytocin could negatively influence the life span of the CL in cows and ewes. Administration of oxytocin to cows (Armstrong and Hansel, 1959) or ewes (Hatjiminaoglou *et al.*, 1979) from days 1 to 7 of the cycle shortened the luteal phase, however, the luteolytic effect of the peptide did not appear to be mediated through altered gonadotropin secretion (Wilks and

Hansel, 1971). In contrast, Milvae and coworkers (1991b) recently reported that administration of oxytocin twice daily for 4 days at all stages of the estrous cycle, or intraluteal infusion of oxytocin from day 2 to 9, had no effect on plasma progesterone concentrations or estrous cycle lengths. The reason underlying the discrepancy between their study and that of Hatjiminaoglou and coworkers (1979) is not clear and was attributed to possible variation among breeds of sheep.

There is more evidence to support rather than oppose a role for oxytocin in the luteolytic process of domestic livestock. Oxytocin stimulates uterine secretion of $PGF_{2\alpha}$ in ewes (Sharma and Fitzpatrick, 1974) and cows (Newcomb *et al.*, 1977) *in vivo* and from endometrial explants *in vitro* (Lafrance and Goff, 1990). Oxytocin levels in peripheral circulation of ewes are elevated during the luteal phase of the cycle (Sheldrick and Flint, 1981) and pulses of oxytocin occur concomitantly with pulses of $PGF_{2\alpha}$ or PGFM during luteal regression in both ewes (Fairclough *et al.*, 1980) and cows (Vighio and Liptrap, 1986). In addition, passive immunization of ewes against oxytocin delays luteal regression (Sheldrick *et al.*, 1980). Collectively, these data suggest that endogenous oxytocin may be involved in the regulation of $PGF_{2\alpha}$ secretion and luteal regression in cows and ewes.

Originally it was believed that oxytocin originated only from the neurohypophysis (posterior pituitary), however, it has since been demonstrated that this peptide is synthesized by large cells of the ovine (Wathes and Swann, 1982) and bovine (Fields *et al.*, 1983) CL and is secreted into the ovarian veins of these species (Flint and Sheldrick, 1982; Walters *et al.*, 1984). In addition, luteal oxytocin accounts for the bulk of oxytocin observed in peripheral circulation during the luteal phase of the cycle in the ewe (Hooper *et al.*, 1986) and to a lesser extent in the cow (Parkinson *et al.*, 1992). Consistent with reported blood

levels of oxytocin during the luteal phase, bovine luteal tissue concentrations of oxytocin increase from day 4 to 8, decline through day 12 and reach a nadir on day 16 of the cycle (Abdelgadir *et al.*, 1987). Parkinson and coworkers (1992) reported similar observations with the exception that luteal concentrations of oxytocin were maximal on day 12 of the cycle.

A stimulatory effect of $PGF_{2\alpha}$ on ovine and bovine luteal oxytocin release has been demonstrated *in vivo* as well as *in vitro*. Systemic administration of a $PGF_{2\alpha}$ analog (cloprostenol) to cows (Shallenberger *et al.*, 1984) and ewes (Flint and Sheldrick, 1982) or infusion of $PGF_{2\alpha}$ into the ovarian arteries of ewes (Lamsa *et al.*, 1989) increased ovarian oxytocin secretion whereas treatment of bovine luteal slices with $PGF_{2\alpha}$ *in vitro* increased the release of oxytocin on day 8 but not days 12 or 16 of the cycle (Abdelgadir *et al.*, 1987). Failure of $PGF_{2\alpha}$ to stimulate oxytocin secretion on day 12 or 16 was attributed to the probable saturation of luteal $PGF_{2\alpha}$ receptors by endogenous prostaglandin. Collectively, these data demonstrate that in domestic ruminants oxytocin is synthesized in the CL and suggest that $PGF_{2\alpha}$ may be the physiological stimulus for luteal oxytocin secretion.

Flint and Sheldrick (1983) proposed a **positive feedback loop hypothesis** to explain the role of luteal oxytocin in the induction of luteal regression. Initially luteal oxytocin was proposed to hasten luteal regression by ensuring that $PGF_{2\alpha}$ was secreted in high amplitude pulses (Flint and Sheldrick, 1982). This premise was expanded to include a positive feedback loop in which luteal oxytocin stimulated uterine $PGF_{2\alpha}$ release which then stimulated the further release of luteal oxytocin and positive feedback continued until oxytocin stores in the CL were depleted and the CL completely regressed.

Recent evidence suggests that the positive feedback loop may commence with uterine release of $PGF_{2\alpha}$ because utero-ovarian levels of $PGF_{2\alpha}$ increase in ewes undergoing spontaneous luteolysis in the face of undetectable levels of oxytocin (Moore *et al.*, 1986). The source of the stimulus that provokes the first episodic release of $PGF_{2\alpha}$ has not been precisely identified, however, oxytocin of neurohypophysial origin is a likely candidate because pulses of $PGF_{2\alpha}$ have been associated with pulses of neurohypophysial and luteal oxytocin in the ewe (Hooper *et al.*, 1986) and small pulses of neurohypophysial oxytocin have been detected in systemic circulation of OVX sheep treated with estradiol (McCracken *et al.*, 1991).

Pulsatile release of $PGF_{2\alpha}$ from the uterus of domestic ruminants near the time of luteal regression appears to occur as series of 5 to 8 discrete pulses every 6 to 8 hr (Silvia *et al.*, 1991) and the interval between pulses is thought to arise from transient uterine (Sheldrick and Flint, 1986) and luteal (Lamsa *et al.*, 1992) refractoriness to oxytocin and $PGF_{2\alpha}$ stimulation, respectively, that is maintained for about 6 hr. Uterine responsiveness to oxytocin is also thought to dictate the first appearance of pulsatile $PGF_{2\alpha}$ secretion, ensuring that luteolysis is initiated at the appropriate time of the estrous cycle. The uterus becomes responsive to exogenous oxytocin only late in the estrous cycle of the ewe (day 14 or 15; Silvia *et al.*, 1992) and cow (days 17 to 20; Lafrance and Goff, 1985; Silvia and Taylor, 1989) and responsiveness is maintained early into the next cycle (Silvia and Taylor, 1989). The mechanism responsible for the regulation of uterine responsiveness to this nonapeptide is presently unknown although Silvia and coworkers (1991) suggested several potential points of regulation. Briefly, uterine responsiveness to oxytocin could be controlled through regulation of the synthesis of $PGF_{2\alpha}$ (Huslig *et al.*, 1979), availability of its

precursor arachidonic acid (Irvine, 1982) or synthesis of the oxytocin receptor (Roberts *et al.*, 1976; Meyer *et al.*, 1988; Jenner *et al.*, 1991). The relative importance of each of these regulatory sites as a determinant of uterine secretory responsiveness to oxytocin at the time of luteal regression is unknown; however, it is clear that negative regulation at any one of these sites could greatly influence $PGF_{2\alpha}$ secretion.

Not surprisingly, both progesterone and estradiol appear to play a role in the regulation of luteal regression in ewes and cows. The two steroids appear to have both stimulatory and inhibitory effects on uterine $PGF_{2\alpha}$ secretion. Administration of progesterone to ewes (Zimbelman *et al.*, 1959) or cows (Loy *et al.*, 1960) early in the cycle results in premature luteal regression presumably by stimulating uterine $PGF_{2\alpha}$ secretion. Similarly, premature withdrawal of progesterone also stimulates $PGF_{2\alpha}$ secretion in ewes (Vincent and Inskeep, 1986) suggesting that progesterone late in the cycle is inhibitory to $PGF_{2\alpha}$ release.

The stimulatory effects of progesterone could be manifested at several different loci within the endometrial cell (Silvia *et al.*, 1991) resulting in increased accumulation of $PGF_{2\alpha}$ precursor (Brinsfield and Hawk, 1973) or increased synthesis of prostaglandin H (PGH) endoperoxide synthase (PGH synthase; cyclooxygenase; arachidonic acid $\rightarrow$ $PGH_2$) mRNA (Eggleston *et al.*, 1990) and protein (Raw *et al.*, 1988). Progesterone could also stimulate $PGF_{2\alpha}$ secretion by increasing uterine sensitivity to oxytocin, however, the effect of progesterone on oxytocin receptor levels is equivocal. Vallet and coworkers (1990) reported that progestogen pretreated (fluorogestone acetate-impregnated intravaginal pessary; day -10 to day 0) OVX ewes receiving exogenous estradiol for 2 days (day 0 and 1) and progesterone for 12 days (day 2 to day 13) had a greater number of endometrial oxytocin

receptors, the day after the last steroid treatment, than similarly treated ewes receiving progesterone for only 5 days (day 2 to day 6). From these data, Vallet and coworkers (1990) suggested that progesterone may act early in the cycle to reduce and late in the cycle to enhance endometrial sensitivity to oxytocin by altering the number of oxytocin receptors. In contrast, several investigators have reported that administration of progesterone to OVX ewes for 12 (Lau *et al.*, 1992) or 9 or 12 (Zhang *et al.*, 1992) days after steroid pretreatment or no pretreatment, respectively, reduced endometrial oxytocin receptor levels on day 14 and days 10 and 13, respectively, compared with control ewes. The differences between the three studies may be attributed to differences in steroid treatment regimens. Interestingly, two of the studies (Vallet *et al.*, 1990; Lau *et al.*, 1992) reported an apparent lack of correlation between uterine PGFM response to exogenous oxytocin and endometrial oxytocin receptor concentrations, suggesting that other factors may be more important in controlling oxytocin-induced $PGF_{2\alpha}$ release.

Progesterone may negatively regulate $PGF_{2\alpha}$ by reducing uterine oxytocin receptor concentrations because the number of endometrial receptors for this nonapeptide increase within 6 hr after progesterone withdrawal (Leavitt *et al.*, 1985). Similarly, Zhang and coworkers (1992) reported increased oxytocin receptor levels in both the endometrium and myometrium of ewes after progesterone withdrawal. Because progesterone is able to inhibit synthesis of estrogen receptors (Koligian and Stormshak, 1977) and the synthesis of oxytocin receptors is an estradiol-dependent process (McCracken, 1980), it seems probable that progesterone could negatively influence uterine responsiveness to oxytocin by inhibiting the synthesis of estradiol receptors, thus impairing the ability of estradiol to induce the synthesis of oxytocin receptors. In the latter stages of the estrous cycle, eventual refractoriness of the

uterus to progesterone, as a result of progesterone receptor down-regulation, might allow estrogen to induce the synthesis of its own receptor and hence the oxytocin receptor, thereby restoring endometrial sensitivity to oxytocin at a time appropriate for luteolysis. The eventual desensitization of the uterus to progesterone, after prolonged exposure to the steroid during the luteal phase, has been suggested to be the critical event that permits uterine oxytocin responsiveness and subsequent secretion of $PGF_{2\alpha}$ (McCracken *et al.*, 1984). The latter assertion is disputed by Vallet and colleagues (1990) because treatment of OVX ewes with progesterone for 12 days increased oxytocin receptor levels and PGFM response to exogenous oxytocin and additional treatment with estradiol on days 12 and 13 failed to increase either response. Further, early withdrawal of progesterone decreased PGFM response to oxytocin, suggesting that progesterone, and presumably its receptor, are required for luteolysis.

Estrogens appear to play a role in the regulation of luteal regression because destruction of ovarian follicles, a major source of estrogens, by irradiation results in prolonged luteal function in ewes ( Hixon *et al.*, 1975) and cows (Villa-Godoy *et al.*, 1981). In addition, estradiol stimulates the synthesis and release of uterine $PGF_{2\alpha}$ in OVX ewes (Ford *et al.*, 1975), however, the magnitude of response to estradiol is enhanced upon previous exposure to progesterone (Barcikowski *et al.*, 1974; Knickerbocker *et al.*, 1986). Further, as was the case with progesterone, estradiol may also exert its effects on $PGF_{2\alpha}$ secretion by altering uterine responsiveness to oxytocin (Vallet *et al.*, 1990; Zhang *et al.*, 1992). Vallet and coworkers (1990) reported that treatment of progestogen-primed (day - 10 to day 0) OVX ewes with estradiol for 2 days (day 0 to day 1), to mimic estrus, significantly reduced the number of endometrial oxytocin receptors on day 7 compared with control ewes

treated with corn oil. In contrast, similarly treated ewes administered progesterone for 5 days (day 2 to day 6) and estradiol for 2 days (day 5 and 6; to mimic early luteal follicular wave), had a greater number of endometrial oxytocin receptors on day 7 compared with ewes receiving progesterone treatment alone. Further, although ewes receiving progesterone for either 10 (day 2 to 11; premature withdrawal of progesterone) or 12 days (day 2 to 13) in combination with estradiol for 2 days (day 12 and 13; to mimic late luteal follicular wave) did not exhibit a greater number of uterine oxytocin receptors on day 14, estradiol treatment significantly enhanced PGFM response to exogenous oxytocin on that day. From these data these researchers suggested that early in the cycle estradiol may act to reduce uterine oxytocin sensitivity thus preventing premature luteal regression and may increase oxytocin responsiveness at midcycle in preparation for luteolysis. In addition, because estradiol significantly enhanced rapid $PGF_{2\alpha}$ secretion in response to oxytocin after prolonged exposure to progesterone, but did not increase oxytocin receptor concentrations over that induced by progesterone alone, it was suggested that progesterone alone can induce oxytocin responsiveness late in the cycle and that estradiol may be more important in initiating the high-amplitude short-duration pulses of $PGF_{2\alpha}$ required for luteolysis. Further, estradiol may act late in the cycle to sustain the luteolytic mechanism after progesterone levels decline because the ability of exogenous oxytocin to provoke a rapid release of $PGF_{2\alpha}$ after premature withdrawal of progesterone was preserved in estradiol-treated but not control ewes.

Zhang and coworkers (1992), suggested that progesterone and estradiol act together to regulate uterine oxytocin receptor levels. Treatment with estradiol and progesterone for 9 or 12 days reduced the concentration of uterine oxytocin receptors in OVX ewes on day

10 or 13, respectively, compared to control ewes treated with progesterone alone. However, in contrast, to the report of Vallet and coworkers (1990) estradiol treatment significantly increased uterine oxytocin receptor levels on day 13 in ewes in which progesterone was prematurely withdrawn (progesterone for 9 days followed by 3 days corn oil) suggesting that increasing levels of estradiol (from developing follicles) and decreasing levels of progesterone (due to luteolysis) at the end of the cycle may contribute to increased uterine responsiveness to oxytocin by increasing the number of oxytocin receptors in the endometrium and myometrium.

From the data presented, it can be concluded that progesterone and estradiol can positively or negatively influence uterine responsiveness to oxytocin. However, because differences in steroid treatment regimens between studies appear to drastically influence uterine oxytocin receptor concentrations and perhaps response to oxytocin, it is difficult to discern precisely, the mechanism(s) through which these two ovarian steroids interact to regulate $PGF_{2\alpha}$ secretion. What does seem clear, is that the initiation of $PGF_{2\alpha}$ release at the end of the cycle and the interval between $PGF_{2\alpha}$ pulses, is probably regulated through uterine responsiveness to oxytocin. Uterine sensitivity to oxytocin may be primarily regulated by the induction or loss of endometrial oxytocin receptors and considerable agreement exists among research groups to support the concept that estradiol and progesterone act early in the cycle to reduce endometrial oxytocin receptor levels thus preventing premature $PGF_{2\alpha}$ release and luteal regression. In contrast, there appears to be two different schools of thought concerning the role of progesterone and estradiol in the regulation of endometrial responsiveness to oxytocin late in the cycle, those who believe that uterine secretion of $PGF_{2\alpha}$ is primarily controlled by the inhibitory effect of progesterone

on the synthesis of estradiol receptors (McCracken, 1980, McCracken *et al.*, 1984; Silvia *et al.*, 1991) and those who believe that prolonged exposure to progesterone during the luteal phase of the cycle induces uterine oxytocin receptors and increased responsiveness (Vallet *et al.*, 1992). Further, it should be noted that there is evidence to suggest that steroidal regulation of $PGF_{2\alpha}$ secretion may be mediated by processes other than those controlling the expression of the oxytocin receptor. Therefore, proper initiation and completion of luteal regression most likely relies on coordinate regulation of a number of cellular processes, in addition to modulation of oxytocin receptor levels, including *de novo* synthesis or release from phospholipid of arachidonic acid ($PGF_{2\alpha}$ precursor) as well as induction of the enzymes requisite for $PGF_{2\alpha}$ synthesis.

## Initiation of Estrus Following Calving

After calving, cows experience a period of anestrus prior to the resumption of regular estrous cycles. In suckled beef cows the duration of postpartum anestrus ranges from 65 to 104 days (Wiltbank, 1970; Casida, 1971) and during this period cows fail to exhibit estrus and do not ovulate. During, the early stages of the postpartum interval, the uterus and endometrium recover from the physical trauma of pregnancy and parturition and return to the normal nonpregnant state. This process of uterine involution is usually complete by 30 days postpartum (pp) and is not thought to influence the length of the anestrous period (Kiracofe, 1980); however, conception rates are reduced in cows that return to estrus and are bred before day 20 pp when the uterus is not completely involuted (Short *et al.*, 1990). Suckling of calves (Smith and Vincent, 1972) and poor nutrition (Wiltbank *et al.*, 1962) are two factors known to prolong postpartum anestrous. In many cows, the

first estrous cycle following calving (Foote and Hunter, 1964; Corah *et al.*, 1974) or early weaning of calves (Odde *et al.*, 1980) is likely to result in the development of a CL with a shorter than normal life span and as a consequence the duration of this cycle is reduced and is typically referred to as a "short cycle". Inadequate luteal function and the occurrence of short cycles have also been observed in ewes at the beginning of the breeding season as they undergo the transition from anestrus to regular estrous cycles (Yuthasastrakosol *et al.*, 1975). In addition, short-lived corpora lutea also occur in ewes and heifers at puberty (Gonzalez-Padilla *et al.*, 1975; Keisler *et al.*, 1983). The characteristics of and physiological basis underlying the occurrence of short-lived CL in women (McNeely and Soules, 1988), cows (Short *et al.*, 1990) and sheep (Hunter, 1991) has been recently reviewed.

***Short Cycles***

In beef cows, the formation of a short-lived CL after calving or early weaning results in a transient rise in systemic levels of progesterone that lasts about 4 to 10 days (Odde *et al.*, 1980; LaVoie *et al.*, 1981) and it has been suggested that the transient increase in this steroid may aid in the initiation of normal estrous cycles (LaVoie *et al.*, 1981). Initial research focused on determining if these CL were short-lived as a result of inadequate gonadotropin secretion, altered follicular development, an inherent defect, or premature release of luteolysin.

Several studies have been conducted using postpartum beef cattle to determine if altered gonadotropin secretion is responsible for the development of the CL with a shorter than normal life span. Garcia-Winder and colleagues (1986) used norgestomet-primed (formation of CL with normal life span) and non-primed (formation of short-lived CL)

postpartum beef cows induced to ovulate by administration of hCG (1,000 IU, i.m.) to determine if cows with short-lived CL had different patterns of gonadotropin secretion as compared with cows having CL with a normal life span. These investigators reported that systemic levels of FSH increased after ovulation but did not differ between cows with short- or normal-lived CL. In addition, mean concentrations and frequency of pulses of LH were not different on days 3 and 5 after hCG treatment in cows with either short- or normal-lived CL. Similarly, Garverick and coworkers (1988) reported that mean concentrations as well as frequency, amplitude and duration of pulses of LH and FSH did not differ on days 2, 4, and 6 after the first postpartum estrus in weaned beef cows having either short or normal estrous cycles. Collectively, these data demonstrate that gonadotropin secretion after CL formation does not differ among suckled (Garcia-Winder *et al.*, 1986) or weaned (Garverick *et al.*, 1988) postpartum beef cows exhibiting normal or short estrous cycles and suggest that short-lived CL are most likely not formed as a result of altered gonadotropin secretion after ovulation.

It has been suggested that short-lived CL may develop as a result of inadequate follicular development prior to ovulation. Garcia-Winder and coworkers (1986) reported no difference in preovulatory concentrations or secretory pattern of FSH in postpartum cows forming short- (nonprogestogen-primed) or normal-lived (progestogen-primed) CL after administration of hCG. In contrast, mean concentration and pulse frequency of LH was greater 5 days prior to hCG treatment in cows forming normal as compared with short-lived CL. Further, systemic estradiol levels were greater the day before hCG treatment in cows developing normal but not short-lived CL. These data and those of others (Garcia-Winder *et al.*, 1987) suggest that progestogen-priming increased secretion of LH and enhanced the

development of the dominant follicle, reflected by increased systemic concentrations of estradiol, resulting in a luteal phase of normal duration. Further, lack of an increase in LH secretion and systemic levels of estradiol in cows developing short-lived CL suggests that follicular development may be impaired prior to the first ovulation after calving as a result of reduced LH secretion. In addition, impaired follicular maturation may result as a consequence of factors other than or in addition to reduced preovulatory secretion of LH because others (Garverick *et al.*, 1988) have reported no differences in gonadotropin secretion prior to ovulation in postpartum cows forming normal or short-lived CL. In support of this concept, progestogen treatment of suckled beef cows (expected to have normal luteal phase) resulted in the development of a large follicle that contained a greater amount of follicular fluid and number of LH receptors in theca and granulosa cells compared with the largest follicle from untreated cows expected to have a short luteal phase (Inskeep *et al.*, 1988). These data suggest that short-lived CL may arise from inadequately developed ovulatory follicles as a result, in part, of reduced follicular sensitivity to LH .

Because maintenance of the CL is dependent upon LH stimulation, Smith and coworkers (1986) conducted an experiment to determine if short-lived CL resulted from differences in the LH signal transduction pathway. These investigators reported that impaired luteal function of short-lived CL did not appear to be due to a reduction in the concentrations of unoccupied or occupied LH receptors, or basal or agonist-activated (LH or guanylimidodiphosphate [Gpp(NH)p]) adenylate cyclase activity. Phosphodiesterase activity (cAMP→5'AMP) was greater in CL anticipated to be short-lived as compared with those having a normal life span, however, the significance of this finding is unclear because plasma (days 1 to 5) and luteal (day 5) progesterone concentrations did not differ between

CL from the two groups. Similarly, Braden and coworkers (1989) reported no difference in the number of LH receptors in ovine CL collected on day 4 or 7 after the first postpartum ovulation as compared with CL collected from normal cyclic ewes on the same days of the estrous cycle.

Copelin and coworkers (1987) conducted an experiment to determine if the reduced life span of the first CL formed after calving was inherent or the result of premature luteolysis. Beef cows were weaned and subjected to hysterectomy or sham surgery (control) on day 23 to 33 pp. The first estrous cycle of control cows, induced by weaning of calves, was significantly shorter than the subsequent cycle ($8.8 \pm .3$ v $20.2 \pm .5$ days). Conversely, CL of the first cycle were maintained in hysterectomized cows until day 20 of the cycle whereupon $PGF_{2\alpha}$ (25 mg, i.m.) was administered and cows returned to estrus within 48 to 72 hr after treatment. Progesterone concentrations on day 0 to 5 of the first cycle were similar between groups, however, after day 5, levels of the steroid decreased in control cows whereas in hysterectomized cows they continued to increase, reaching a plateau on day 12 of the cycle. Plasma concentrations of LH and estradiol, several days prior to estrus, were similar for both control and hysterectomized cows. Similar results have been reported for the ewe (Southee *et al.*, 1988). Collectively, these data suggest that in weaned cows, the CL of the first postpartum estrous cycle is not inherently short-lived as a result of an impaired ability to respond to gonadotropin but rather the uterus exerts a luteolytic effect on the CL early in the luteal phase (day 6), resulting in a short cycle.

Although the short-lived CL appears to result from premature luteolysis, this condition might be manifested through increased luteal sensitivity to and(or) the premature release of uterine $PGF_{2\alpha}$. Copelin and colleagues (1988) investigated this latter issue using

hysterectomized suckled beef cows and reported that CL anticipated to have a short life span were not more sensitive to exogenous $PGF_{2\alpha}$ (10 mg, i.m). Similar results have been reported for ewes (Braden *et al.*, 1989). A subsequent study further strengthened the concept that short-lived CL result from premature release of luteolysin, presumably $PGF_{2\alpha}$, because prepartum or postpartum immunization of mature beef cows against $PGF_{2\alpha}$ extended the life span and secretion of progesterone of CL anticipated to be short-lived (Copelin *et al.*, 1989). In addition, oxytocin-induced uterine $PGF_{2\alpha}$ secretion, as estimated by PGFM concentration, was greater in cows having a short as compared with normal luteal phase and response to oxytocin on day 5 of a short cycle was similar to that observed on day 16 of a normal estrous cycle, suggesting that the uterus is more sensitive to oxytocin earlier in the cycle in cows having short-lived CL (Zollers *et al.*, 1989). The concept that subnormal luteal function arises as a consequence of the premature release of $PGF_{2\alpha}$ is also supported by the results of recent *in vitro* experiments (Zollers *et al.*, 1991) in which endometrial explants (day 5 pp) from postpartum beef cows anticipated to have a short estrous cycle released more total PGF (sum of $PGF_{1\alpha}$, $PGF_{2\alpha}$ and PGFM) as compared with explants from cows expected to have a normal cycle length. Treatment of endometrial explants with oxytocin (100 or 1000 pmol) *in vitro* yielded similar results to those obtained *in vivo* (Zollers *et al.*, 1989). Explants from cows expected to have a short cycle responded to oxytocin treatment with significant increases in total PGF released whereas similarly treated explants from cows anticipated to have estrous cycles of normal length demonstrated no increase in total PGF secretion in response to the nonapeptide (Zollers *et al.*, 1991). In contrast, others (Cooper *et al.*, 1991) have suggested that oxytocin may not control the premature release of $PGF_{2\alpha}$ observed in cows with short-lived CL because on days 4 to 9 of the estrous cycle

systemic concentrations of the nonapeptide were not different between cows having CL with a short or normal life span.

Recently, it has been suggested that prostaglandins of luteal origin may also play a role in the premature regression of CL after calving (Hu *et al.*, 1990). Lactating beef cows between days 35 and 40 pp were treated with progestogen implants (norgestomet) for 9 days (expected to develop CL with normal life span) or received no implant (anticipated to develop short-lived CL) and all cows were induced to ovulate with hCG (1000 IU, i.m.). Corpora lutea were collected on day 6 after hCG treatment and were subjected to enzymatic dispersion and *in vitro* incubation with various concentrations of calcium ionophore (A23187; 0, 0.05, 0.5, and 5.0 $\mu$M) to stimulate prostaglandin synthesis. Although basal and ionophore-stimulated production of progesterone did not differ between CL anticipated to be normal- or short-lived, basal production of $PGF_{2\alpha}$, PGI and $PGE_2$ was greater in subnormal (short-lived) as compared with normal CL. In response to the lowest concentration of ionophore, $PGF_{2\alpha}$ was significantly elevated in subnormal CL, whereas, PGI and $PGE_2$ synthesis was increased in similarly treated normal CL. In contrast, addition of the highest concentration of ionophore reduced prostaglandin synthesis in subnormal but not normal CL. The ratio of PGI to $PGF_{2\alpha}$ was greater in normal CL than subnormal CL, irrespective of ionophore dose, whereas, the ratio of $PGE_2$ to $PGF_{2\alpha}$ was greater in normal as compared with subnormal CL for untreated cells and at the lowest dose of ionophore. Collectively, these data suggest that short-lived CL preferentially synthesize $PGF_{2\alpha}$, leading to a shift in the ratio of luteotropins (PGI, $PGE_2$) to luteolysin ($PGF_{2\alpha}$) which may play a role in the premature demise of the first CL after cal

To date several mechanisms have been suggested to play a role in the formation of short-lived CL, including inadequate follicular development, premature release of uterine $PGF_{2\alpha}$ and enhanced luteal $PGF_{2\alpha}$ synthesis. Clearly more research is required to further delineate the mechanism(s) involved in regulating the length of the luteal phase of the first CL formed after calving or in response to early weaning, however, it seems likely that short cycles occur as a result of the cumulative effects of these and perhaps other yet unknown mechanisms.

## The Hypothalamic-Hypophysial Axis: Functional Aspects During the Postpartum Period

Initial research conducted to investigate the mechanism(s) underlying postpartum anestrus in domestic ruminants revealed that this period of ovarian acyclicity did not result from an inability of the ovary to respond to gonadotropins (Casida *et al.*, 1943). Subsequent research revealed that systemic levels of LH were low during gestation and following parturition in cows and sheep and that ovulation and the return to normal estrous cycles was preceded by an increase in the pulsatile release of this gonadotropin (Arije *et al.*, 1974; Restall and Starr, 1977; Humphrey *et al.*, 1983). Consequently, it was hypothesized that reduced secretion of LH was responsible for the period of acyclicity following parturition and research was initiated to determine the mechanism(s) underlying the regulation of LH secretion in postpartum cows and ewes.

### Potential Regulatory Sites of LH Secretion

Because pulsatile LH secretion is induced by the pulsatile release of GnRH from the hypothalamus, secretion of LH might be negatively influenced by factors acting on the hypothalamus to reduce the synthesis or secretion of GnRH and(or) on the pituitary to reduce its sensitivity to GnRH or decrease the synthesis of gonadotropin. Therefore, research was conducted to determine if the function of one or more of these aspects of the hypothalamic-hypophysial axis was suppressed during pregnancy and after parturition, thus resulting in the suppression of systemic LH concentrations observed during the postpartum period. Function of the hypothalamic-hypophysial axis during the postpartum period in ewes and cows has been reviewed (Nett, 1987).

*Hypothalamic Content and Secretion of GnRH*

Concentration of GnRH in the hypothalamus proper (HYP), preoptic-suprachiasmatic area (POA) and median eminence (ME) of acyclic ewes on days 1 and 11 and of cyclic ewes after day 30 postpartum have been reported (Moss *et al.*, 1980). No significant differences in GnRH concentration in HYP, POA or ME were observed on any of the three days, however, concentrations of the decapeptide in the ME were significantly greater than those in the HYP and POA, which did not differ from one another, on all days examined. Moss and coworkers (1985) reported similar findings for suckled beef cows in which the quantity of GnRH in the HYP, POA and ME did not differ on days 5, 10, 20 and 30 postpartum. Because hypothalamic content of GnRH did not vary significantly during the postpartum period of cows and ewes and did not differ between acyclic and cyclic ewes, it was suggested that the quantity of GnRH in the hypothalamus was sufficient to stimulate pituitary gonadotrophs.

Despite strong evidence provided by the two previously described experiments to support the hypothesis that reduced hypothalamic content of GnRH is not responsible for the reduced systemic concentrations of LH in postpartum cows and sheep, there is also evidence to suggest that hypothalamic content of the decapeptide may be reduced early in the postpartum period of cows. Allrich and coworkers (1985) used a superfusion technique to evaluate the *in vitro* release of GnRH from pituitary stalk-median eminence (SME) obtained from postpartum suckled beef cows. Exposure of bovine SME to a 10-min pulse of high $K^+$ (60 mM) Krebs-Ringer phosphate medium stimulated GnRH secretion above basal levels and the maximum induced peak release of the decapeptide (pg GnRH $\cdot$ ml medium$^{-1}$ $\cdot$ mg SME$^{-1}$) was significantly greater on day 5 ($2.38 \pm .25$) than on day 20 ($.92 \pm .23$)

postpartum. Because *in vitro* $K^+$-induced GnRH release was enhanced on day 5 but not on day 20, these researchers suggested that *in vivo* GnRH release is suppressed shortly after parturition resulting in elevated stores of the decapeptide on day 5 which, by day 20 postpartum, return to normal, as the suppression of release is reduced.

More recently, Leshin and coworkers (1992a) reported morphological differences among GnRH neurons from postpartum and cycling beef cows. Forebrains of early (days 10 to 16) and mid-postpartum (days 33 to 43) cows contained a population of GnRH neurons with significantly shorter and fewer dendritic processes than those from cyclic cows. Analysis of serial sections of ME for GnRH immunoreactivity revealed that, in this tissue, the percentage of area occupied by immunostained fibers was less in early compared with mid-postpartum and cyclic cows. Reduction in the percentage area of GnRH immunostaining neurons observed in the ME of early postpartum cows suggests that the hypothalamic content of GnRH may be reduced early after parturition but returns to normal midway through the postpartum interval. Collectively, these data are consistent with the hypothesis that in the postpartum cow, a population of GnRH neurons undergo structural and biosynthetic modifications that may facilitate the return of normal estrous cycles.

Although data concerning hypothalamic concentration of GnRH in postpartum cows are equivocal, it is clear that measurable levels of GnRH are present early in the postpartum period and its release can be stimulated *in vitro*. Because the quantity of GnRH was reduced after parturition in some cows, and this reduction may result in insufficient stimulation of gonadotrophs, the possibility that this component of the hypothalamic-pituitary axis contributes to the reduction in systemic LH levels after parturition cannot be completely ruled out.

Assuming that GnRH content is adequate, reduced LH synthesis and secretion during the postpartum interval could be explained by a reduction in the frequency or amplitude of GnRH pulses released from the hypothalamus. It appears that GnRH is necessary for the synthesis of LH because the quantity of mRNA for the $\alpha$ and $\beta$-subunits of LH in ovariectomized (OVX) ewes decreases after hypothalamic-pituitary disconnection (Hamernik *et al.*, 1986). Unfortunately, because of the difficulty associated with sampling hypothalamic-hypophysial portal blood, GnRH secretory patterns have not yet been characterized in late pregnant or postpartum ewes and cows. Thus it remains to determined if reduced secretion of GnRH contributes to the suppression of LH secretion during the postpartum period of ewes and cows.

***Pituitary Sensitivity to GnRH***

Sensitivity of the pituitary to GnRH is another aspect of the hypothalamic-hypophysial axis that might influence LH secretion in postpartum cows and ewes. Pituitary sensitivity would be decreased by a reduction in the number of receptors for GnRH or a postreceptor defect. As a result, the number of pituitary receptors for GnRH was determined in pregnant and postpartum ewes (Crowder *et al.*, 1982) and cows (Moss *et al.*, 1985; Leung *et al.*, 1986; Nett *et al.*, 1988; Rahe *et al.*, 1988). Crowder and coworkers (1982) reported that after parturition, concentration of GnRH receptors decreased with time (days) after parturition in ewes. Receptor levels were greater on days 1 and 11 versus days 22 and 35 postpartum, suggesting that pituitary sensitivity to GnRH was not reduced early after lambing. Receptor concentrations in late pregnancy (approximately day 150) were similar to those on days 22 and 35 postpartum and receptor numbers during pregnancy and after parturition never declined below those observed in OVX ewes, in which LH secretion

is chronically elevated. Further, previous research has demonstrated no difference in GnRH receptor concentrations in the pituitaries of OVX and luteal phase ewes (Moss *et al.*, 1981). Collectively, these data suggest that low systemic concentrations of LH in postpartum ewes, most probably, are not the result of a reduction in the number of GnRH receptors in the anterior pituitary.

Numbers and affinity constants of GnRH receptors in the pituitaries of suckled beef cows on days 5, 10, 20 and 30 postpartum have been reported (Moss *et al.*, 1985). Although neither the affinity constant nor number of receptors for GnRH differed among the days examined, the combined total number of receptors on days 5 and 10 was significantly greater than that on days 20 and 30. Concentrations of GnRH receptors in luteal phase cows were similar to those observed in postpartum cows on days 20 and 30, but were lower than those on days 5 and 10. Similarly, Leung and coworkers (1986) reported that the numbers and affinity constants of GnRH receptors in pituitary glands of suckled anestrous beef cows on days 7, 14, 28, 42 and 56 and cyclic beef cows on days 42 and 56 postpartum did not differ significantly. Collectively, these data support the hypothesis that number and affinity of GnRH receptors does not limit the ability of the bovine pituitary to release LH during the postpartum interval. In contrast, Nett and coworkers (1988) reported that GnRH receptor levels in the pituitaries of suckled beef cows were minimal on day 1, attained maximum levels on day 15 and subsequently declined through day 45 postpartum. These authors suggested that the rise in GnRH receptors may increase the sensitivity of the pituitary to the decapeptide, possibly provoking enhanced synthesis of LH and subsequent restoration of pituitary stores by day 30 postpartum. In addition, it was suggested that although there were

fewer receptors for GnRH on day 1 postpartum, the number of receptors present were most likely adequate for gonadotroph stimulation.

Further indirect evidence has been reported that supports the hypothesis that pituitary GnRH receptor concentrations are sufficient to support LH synthesis and secretion in late pregnant and early postpartum cows (Rahe *et al.*, 1988). Receptor levels were greatest on day 120 of pregnancy but had diminished by day 200 and 275 of pregnancy and remained low for 30 to 48 hr after calving. During the latter stages of pregnancy and shortly after parturition GnRH receptor levels were similar to those observed in cyclic beef cows on day 3 of the estrous cycle. Therefore, because LH secretion ($7.8 \pm .5$ pulses LH/8 hr) was not impaired in cyclic cows, it seems very likely that an ample concentration of GnRH receptors exists late in pregnancy and after parturition to induce the synthesis and secretion of LH.

Postpartum pituitary sensitivity to GnRH has been evaluated *in vivo* (Fernandes *et al.*, 1978) and *in vitro* using enzymatically dispersed pituitary cells (Moss *et al.*, 1985) and pituitary slices (Leung *et al.*, 1986) in short-term culture. Fernandes and coworkers (1978) demonstrated that plasma concentrations of LH are increased within 5 min after GnRH injection (100 $\mu$g, i.m.) in dairy cows on days 3, 10, 20, 30, and 40 postpartum. However, although the pituitary was able to respond to GnRH as early as day 3, LH response to GnRH was not fully restored until after day 10 postpartum suggesting that reduced pituitary stores of LH might be limiting the response to GnRH early after calving. Similarly, LH response to GnRH (200 $\mu$g, i.v.) was significantly greater on day 14 than on day 1 in postpartum beef cows (Jaeger *et al.*, 1987).

Results from *in vitro* experiments also suggest that the pituitary is responsive to GnRH early in the postpartum period. Pituitary cells prepared from cows on days 5, 10,

20 and 30 postpartum did not differ with respect to basal or GnRH-induced LH release, however, GnRH did stimulate LH release above basal levels on all days examined (Moss *et al.*, 1985). In addition, similarly prepared cells from luteal phase cows released more LH in response to GnRH than did those from cows on day 5, but not day 10, 20, or 30, suggesting that pituitary stores of LH are comparable to those observed in cyclic cows by day 10 postpartum. Similarly, treatment of pituitary slices, obtained from cows between days 7 and 42 of the postpartum period, with GnRH *in vitro* evoked LH release above basal levels (Leung *et al.*, 1986). In addition, a positive correlation between pituitary content of LH and basal ($r$=.76) and GnRH-induced ($r$=.75) LH release *in vitro* was also reported, further supporting the concept that the bovine pituitary is sensitive to GnRH early after parturition and that response to GnRH is dependent upon pituitary content of the gonadotropin.

Collectively, the majority of these experiments demonstrate that number of GnRH receptors in the anterior pituitary probably does not limit the ability of gonadotrophs to synthesize and secrete LH during the postpartum interval of cows and ewes. Further, although response to GnRH is reduced early in the postpartum period, LH secretion is still provoked by the decapeptide, both *in vivo* and *in vitro,* suggesting that pituitary sensitivity is not diminished by a postreceptor defect at this time.

***Pituitary Content of LH***

Pituitary LH content is severely depleted late in pregnancy and early in the postpartum period in both cows (Nalbandov and Casida, 1940; Moss *et al.*, 1985; Nett *et al.*, 1988; Rahe *et al.*, 1988) and sheep (Chamley *et al.*, 1976; Jenkin *et al.*, 1977; Crowder *et al.*, 1982). In ewes, pituitary concentration of LH was lowest during late pregnancy ( ~ day

150) and on day 1 postpartum compared to days 11, 22 or 35 after parturition (Crowder *et al.*, 1982). In addition, pituitary content of LH was highly correlated with GnRH-induced LH release *in vivo* ($r$=.90, Jenkin *et al.*, 1977; $r$=.97, Crowder *et al.*, 1982). In cows, pituitary content of LH was lowest on day 275 of pregnancy and day 2 postpartum (Rahe *et al.*, 1988). Others have reported that pituitary content is reduced from days 5 to 20 (Moss *et al.*, 1985) or 1 to 15 ( Nett *et al.*, 1988) postpartum but is restored to levels observed in luteal phase cows by day 30 postpartum. Collectively, these data demonstrate that pituitary stores of LH are reduced beginning late in pregnancy, remain low early after parturition, and increase with increasing days postpartum.

Because pituitary content of LH is highly correlated with GnRH-induced LH release *in vivo* (Jenkin *et al.*, 1977; Crowder *et al.*, 1982) and a constant percentage of LH was released from dispersed pituitary cells in response to a maximal dose of GnRH *in vitro* (Moss *et al.*, 1980), it seems likely that reduced pituitary content of LH, rather than decreased pituitary sensitivity to GnRH, is responsible for the low systemic levels of LH observed during pregnancy and after parturition in domestic ruminants. It should be remembered, however, that while pituitary content of LH is probably the major determinant restricting the resumption of estrous cycles early after parturition, the possibility that hypothalamic content and secretion of GnRH may also negatively influence LH synthesis or release during the postpartum period cannot be dismissed with great certainty.

**Steroid Regulation of LH Synthesis and Secretion**

Reduced pituitary and systemic concentrations of LH, during the latter stages of pregnancy and early in the postpartum interval of cows and sheep, are associated with high

peripheral concentrations of estradiol and progesterone (Stabenfeldt *et al.*, 1972; Arije *et al.*, 1974; Carnegie and Robertson, 1978). These steroids have been demonstrated to play a role in the regulation of LH synthesis and secretion during the estrous cycle of these and other species and estradiol receptors are present in the hypothalamus and anterior pituitary of pregnant and postpartum sheep (Wise *et al.*, 1986) and postpartum cows (Nett *et al.*, 1988). Thus, it seems likely that estradiol and progesterone might play a role in regulating peri- and postparturient LH synthesis and secretion.

Moss and coworkers (1981) conducted an experiment to determine if gonadal steroids could alter pituitary and serum LH concentrations in ewes. Ovariectomized ewes received injections (s.c.) of progesterone (16 mg), estradiol (700 $\mu$g), progesterone plus estradiol or no steroid twice daily for 3 wk. Treatments were designed to produce serum steroid concentrations comparable to those observed during the latter stages of gestation. Progesterone treatment alone suppressed serum levels of LH to those observed in intact ewes during the luteal phase but did not significantly reduce pituitary content of the gonadotropin from that of OVX control ewes. In contrast, estradiol alone or in combination with progesterone significantly reduced both serum and pituitary concentrations of LH compared with those observed in OVX control ewes. Anterior pituitary receptors for GnRH were significantly increased in ewes receiving estradiol alone or in combination with progesterone compared with OVX control, intact luteal phase or progesterone-treated ewes; however, content of GnRH in the HYP, POA and ME did not differ among treatment groups. These data demonstrate that chronic administration of estradiol and(or) progesterone to OVX ewes reduces pituitary and(or) serum LH concentrations similar to that reported for pregnant ewes. Additionally, because the steroids had differential effects, it is probable that estradiol

and progesterone regulate LH synthesis and secretion through different pathways. Interestingly, estradiol-mediated inhibition of LH appears to occur in the face of increased pituitary sensitivity to GnRH because estradiol treatment increased the number of receptors for GnRH in the anterior pituitary.

These data can be interpreted to suggest that progesterone most likely acts at the level of the hypothalamus or at higher neural centers to reduce GnRH secretion, and perhaps synthesis, but probably has no direct effect on LH synthesis because GnRH receptor number and pituitary content of LH were unaffected by treatment with this steroid. In support of this concept, administration of progesterone to OVX ewes profoundly inhibited GnRH and LH secretion (Karsch *et al.*, 1987) but failed to alter pituitary content of mRNA for the subunits of LH (Hamernik *et al.*, 1987). In contrast, estradiol may act directly at the level of the pituitary to inhibit the synthesis and secretion of LH and(or) at the hypothalamus or higher brain centers to inhibit the secretion of GnRH. This latter hypothesis is supported by the observation that estradiol almost completely abolished the occurrence of GnRH pulses in OVX ewes treated during the non-breeding season (Karsch *et al.*, 1987).

The mechanism whereby estradiol decreases pituitary concentration of LH in OVX ewes appears to involve decreased synthesis of mRNA for both the $\alpha$ and $\beta$ subunits of the gonadotropin because chronic treatment of OVX ewes with estradiol reduced $\alpha$- and $\beta$-subunit mRNA in the anterior pituitary by 85 and 98 percent, respectively (Nilson *et al.*, 1983). Similar reductions in mRNA for the subunits of LH has been reported for ewes during gestation and early after parturition (Wise *et al.*, 1985) and presumably this is the case for cows. Direct regulation of LH subunit synthesis by estradiol seems likely because this steroid has been reported to inhibit transcription of the $\alpha$-subunit gene in ovine pituitary

cells *in vitro* (Phillips *et al.*, 1988) and the promoter region of the rat LH $\beta$ gene contains a high affinity binding site for estrogen receptor (Shupnik *et al.*, 1989). Further, estrogen may directly regulate transcription of some genes that lack a functional estrogen response element (ERE) because chronic administration of estradiol (14 days) to OVX transgenic mice harboring an $\alpha$-subunit promoter construct that lacked a functional ERE (1500 bp of the proximal 5′ flanking region and promoter of the human $\alpha$-subunit gene linked 5′ to the bacterial chloramphenicol acetyltransferase gene; H$\alpha$CAT) attenuated both serum LH concentration and pituitary CAT activity, indicating a reduction in the expression of the transgene (Keri *et al.*, 1991). These data suggested that the negative effects of estradiol on human $\alpha$-subunit transcription occurred through an indirect mechanism that was pituitary specific. Additional studies using transgenic mice containing a bovine $\alpha$-subunit construct (314 bp of the 5′ flanking region of the bovine $\alpha$-subunit linked to the CAT gene; B$\alpha$CAT) revealed similar responses to estradiol treatment (Keri *et al.*, 1991). Although it was not determined if the bovine construct contained an ERE, the authors proposed that estradiol likely regulates both the human and bovine LH $\alpha$-subunit genes through similar mechanisms because the proximal 314 bp of the 5′ flanking region of the two genes share considerable sequence homology (85%).

Estradiol-mediated regulation of the $\beta$-subunit of LH may also occur directly at the pituitary. Positive regulation of rat LH $\beta$-subunit gene expression by estradiol appears to occur through the binding of the steroid-receptor complex to a putative ERE located in the promoter regulatory region of the gene (Shupnik *et al.*, 1989). Pituitary $GH_3$ cells, which express estrogen receptors, were transfected with DNA constructs in which portions of the 5′-flanking region of the rat LH$\beta$ gene were inserted next to the herpes simplex virus

thymidine kinase (tk) promoter fused to the CAT reporter gene (LHβ-tkCAT). Treatment of the transfected cells with estradiol increased CAT expression thus demonstrating that the rat LHβ gene can respond directly to estrogen stimulation in $GH_3$ cells. Regulation of CAT expression was always positive and no biphasic response due to duration of treatment or dose of estrogen was observed, suggesting that negative regulation of the LHβ gene might involve other trans-acting factors. Further, the putative LHβ ERE was located about 1.1 kb upstream from the transcriptional start site and shares a high degree of sequence similarity to the ERE in the rat prolactin gene. The presence of an ERE in the bovine or ovine LH β gene has not yet been reported; however, if the expression of the β-subunit in these species was regulated in a manner comparable to that which may occur in the rat, the reduction in LHβ mRNA observed late in gestation and early in the postpartum period might possibly be explained by a reduction in the positive feedback of estradiol on LH synthesis. This hypothesis is supported by the finding that, in the ewe, anterior pituitary concentrations of estradiol receptors are decreased during gestation, remain low during the early postpartum period and increase with increasing days postpartum, thus paralleling the rise in pituitary content of LH (Wise *et al.*, 1986).

Collectively, these experiments suggest that expression of the genes for the α- and β-subunit of LH may be directly regulated by estradiol at the pituitary; however, the mechanism of transcriptional regulation for the two genes appears to be profoundly different (ERE versus non-ERE). Clearly, further research is warranted to determine the exact mechanism whereby estradiol directly regulates the expression of the α and β subunits of LH in domestic ruminants if we are to understand how this steroid may regulate gonadotropin synthesis during the postpartum period and other reproductive states.

Although a growing body of evidence supports the premise that chronic exposure to elevated concentrations of estrogen, as occurs during pregnancy, may suppress LH synthesis and secretion through a direct action of the steroid on pituitary gonadotropes, it has also been suggested that the primary locus of estrogen action is at the hypothalamus and that the inhibitory effects of the steroid are mediated indirectly through suppression of GnRH secretion. Using the OVX-hypothalamo-pituitary disconnected (OVX-HPD) ewe as their animal model, Mercer and coworkers (1988) conducted an experiment to determine the if the primary regulatory site of estrogen action on pituitary LH $\beta$-subunit mRNA levels resided in the hypothalamus or pituitary. Ewes received no treatment (OVX-HPD), estradiol ($E_2$) implant (OVX-HPD+$E_2$), pulsatile infusion of GnRH (OVX-HPD+GnRH) or estradiol implant plus GnRH infusion (OVX-HPD+GnRH+$E_2$) beginning at the time of HPD, approximately 1 month after ovariectomy. Estradiol treatment (to provide plasma estradiol levels of $\sim$3 pg/ml) and GnRH infusion (250 ng delivered over 6 min every 2 hr into the jugular vein) were initiated at the time of HPD and continued for 1 wk whereupon the ewes were sacrificed. Blood samples were collected from ewes receiving GnRH at -5, +15, +30, and +45 min relative to the start of a pulse, whereas, one sample was collected from each of the other ewes at the time of sacrifice.

Plasma LH levels were nondetectable in OVX-HPD and OVX-HPD+$E_2$ ewes, low in ovary intact ewes ($1.5\pm.1$) and elevated in OVX ewes ($6.0\pm0.4$ ng/ml). Pulsatile infusion of GnRH increased systemic LH concentrations in OVX-HPD and OVX-HPD+$E_2$ and mean LH pulse amplitude ($7.0\pm1.4$ and $6.9\pm1.2$ ng/ml, respectively) was similar for both groups. The effect of the various treatments on pituitary LH$\beta$ mRNA levels was determined by hybridizing $^{32}$P-labelled rat LH$\beta$ cDNA to a northern blot of total anterior

pituitary RNA (10 $\mu$g) from individual ewes in each treatment group and results were expressed as a percentage of the value determined for ovary intact control ewes. As anticipated, ovariectomy increased (153% of control) and OVX-HPD decreased (15% of control) LH$\beta$ mRNA levels in the pituitaries of ewes. Administration of estrogen for 1 week failed to further decrease the levels of LH$\beta$ mRNA (29% of control) as would be expected if estrogen were acting primarily at the pituitary to inhibit LH synthesis. In contrast, LH$\beta$ mRNA levels in OVX-HPD ewes treated with GnRH alone (196% of control) or in combination with estradiol (169% of control) were similar to or greater than those of OVX ewes. Because estradiol did not reduce mRNA levels beyond that of untreated OVX-HPD ewes and GnRH increased LH$\beta$ mRNA levels in the presence or absence of the steroid, these investigators concluded that the dramatic reduction of LH$\beta$ mRNA observed in estradiol-treated hypothalamo-pituitary intact ewes (Nilson *et al.*, 1983) was not a direct effect of estrogen on the pituitary, but rather the result of estrogen action upon the hypothalamus to reduce GnRH secretion. Others have reported similar effects of HPD and subsequent GnRH replacement on pituitary quantity of mRNA for both the $\alpha$ and $\beta$-subunits of LH in OVX ewes (Hamernik and Nett, 1988).

Gonadal steroids have been demonstrated to regulate pro-GnRH gene expression in individual neurons in the brains of both male and female rats (Toranzo *et al.*, 1989), thus, providing further support for a direct action of estrogen, and progesterone, at the hypothalamus. Immunohistochemical and *in situ* hybridization techniques were used to localize immunoreactive GnRH and pro-GnRH mRNA on serial sections of rat brain. As expected, neuronal expression of pro-GnRH mRNA and immunoreactive GnRH was co-localized and was observed, in decreasing order of intensity, in the POA, anterior

hypothalamus, diagonal band of Broca and ventral septal area. Administration of estradiol to OVX rats (.25 μg, twice daily for 14 days) attenuated the increase in pro-GnRH mRNA levels observed in untreated OVX control rats. Similar treatment of OVX rats with progesterone (2 mg, twice daily for 14 days) resulted in a 25 percent reduction in pro-GnRH mRNA levels compared with untreated OVX rats. Concomitant treatment with progesterone and estradiol decreased mRNA levels by 55 percent. These data clearly demonstrate that, in the rat, both estrogen and progesterone can reduce pro-GnRH mRNA accumulation in the hypothalamus. Unfortunately, in this experiment, it could not be determined if the decrease in mRNA accumulation occurred in response to a direct action of the steroids on GnRH neurons or, indirectly, as a result of steroid-induced modulation in pulsatile GnRH secretion. The latter case is more probable because GnRH neurons appear to lack receptors for estradiol and progesterone (Shivers *et al.*, 1983; Karsch and Lehman, 1988; Fox *et al.*, 1990).

To date, evidence accumulating in the scientific literature strongly supports the concept that resumption of regular estrous cycles after parturition in cows and sheep is initially suppressed because pituitary stores of LH are depleted. Pituitary content of LH is reduced late in pregnancy as a result of negative feedback effects of gonadal steroids on LH synthesis. The negative effects of progesterone appear to be mediated at the level of the hypothalamus or higher brain centers to reduce the secretion of GnRH. On the other hand, the inhibitory actions of estradiol appear to be more complex because this steroid seems to alter GnRH secretion from the hypothalamus, as well as, having a direct effect on pituitary gonadotrophs.

A two-phase model describing the recovery of the hypothalamic-hypophysial axis from the negative feedback effects of high systemic concentrations of progesterone and estradiol during pregnancy of sheep and cows has been proposed (Nett, 1987). In this model, the negative effects of progesterone and estradiol during gestation result in a reduction in the synthesis of LH by the anterior pituitary, thus depleting pituitary stores and reducing basal release of the gonadotropin during pregnancy and after parturition. Phase one of the recovery lasts from 2 to 5 wk after parturition and is, presumably, characterized by infrequent releases of GnRH from the hypothalamus. Frequency of GnRH pulses is sufficient to stimulate LH synthesis, but does not greatly increase the secretion, and allows LH stores to be replenished. Phase two begins as pituitary LH content attains normal levels. At this time, secretion of LH increases and the pulses are of sufficient magnitude to stimulate ovarian follicular development. As follicles grow, estradiol is released and subsequently stimulates the synthesis of its own receptor in the hypothalamus and pituitary. The gradual rise in systemic concentrations of estradiol, coupled with the increasing sensitivity of the hypothalamic-pituitary axis to the steroid, invokes a positive feedback loop in which estradiol increases the frequency of LH pulses, provoking final maturation of the ovulatory follicle, subsequent ovulatory surge of LH and the resumption of normal ovarian cycles.

# Factors Affecting the Postpartum Interval of Beef Cows

Because the average duration of gestation in cows is about 281 days, it is vital that the producer use management practices that will minimize the duration of the anestrous period in his herd, thus, maximizing the opportunity for cows to breed and become pregnant during a well defined breeding period. Cows that become pregnant early during the breeding season are more likely to achieve the production goal of weaning one calf each year, whereas, cows that do not become pregnant, or conceive late in the breeding season, represent economic loss to the producer.

Duration of the postpartum period is affected by numerous factors that include breed, parity, dystocia, season, nutrition and suckling (for review, see Short *et al.*, 1990). Nutrition and suckling are considered the two most important factors that influence the length of the postpartum interval, however, in both cases management procedures have been developed to reduce their impact on reproductive efficiency. In contrast, the effect of season of calving on postpartum reproduction is less well understood and is potentially more difficult to circumvent because altering breeding and calving seasons may be economically unfeasible or otherwise impractical. Other minor factors that influence postpartum anestrus, such as breed, parity and dystocia are more easily managed through various culling and breeding practices.

## Nutrition

Almost 60 years ago, nutrition was shown to influence ovarian activity in ewes (Clark, 1934) and this finding was subsequently extended to include sows (Robertson *et al.*,

1951) and cows (Bond *et al.*, 1958). Not surprisingly, nutrition was also found to affect the return to estrus after calving in beef cattle, and it is now generally accepted that undernutrition prior to and(or) after parturition extends the postpartum interval in this species (for review, see Randel, 1990).

Wiltbank and coworkers (1962) conducted an experiment to determine the effects of pre- and postcalving energy level on the occurrence of estrus and conception rate in postpartum Hereford cows. Cows were assigned to one of four treatment groups designated as high-high, high-low, low-high and low-low, referring to the pre- and postcalving energy level. A higher percentage of cows had resumed cycling by 90 days post-calving and estrus was observed earlier after calving in cows on a high plane of nutrition pre-calving than those on the low energy pre-calving diet. Further, cows receiving the low energy pre-calving diet appeared to overcome some of the detrimental effects of undernutrition if they received the high energy diet post-calving because a majority of the cows (85%) in the low-high group had begun cycling by 90 days postpartum, however, the mean length of postpartum interval (65 days) was significantly greater in this group compared with the High-High (48 days), High-Low (43 days) and Low-Low (52 days) groups. Although not significant statistically, conception rates at first service were lower for cows receiving the low energy diet post-calving than those receiving the high energy diet, suggesting that postpartum level of nutrition may influence subsequent conception rates. The mechanism whereby undernutrition increased the postpartum interval in cows was not determined, however, the authors speculated that feeding cows low levels of energy most likely reduced gonadotropin secretion thus impairing follicular development and the return to estrus. Clearly, these results demonstrate that pre- and postcalving energy level can alter reproductive performance in

postpartum beef cows. Feeding low levels of energy to cows prior to calving increased the duration of the postpartum interval, whereas, feeding low levels of energy after calving reduced conception rate at first service.

Echternkamp and coworkers (1982) demonstrated a direct effect of nutrition on the secretion of LH in suckled postpartum beef heifers. Heifers, entering the last trimester of pregnancy, were maintained on a low (100% of the total protein requirement and 70% of the energy requirement for pregnancy and 60% of the energy requirement for early postpartum lactation) or high (100% of the requirement for pregnancy and lactation) planes of nutrition through the second week after calving at which time heifers on the low plane of nutrition were switched to the high plane. To evaluate the effect of diet on the releasable pool of LH, one-half of the heifers in each group were injected with estradiol (10 mg, i.m.) at either 2 or 4 wk postpartum. Maximum LH response to estrogen, but not pre-injection concentrations, were influenced by diet and weeks postpartum. Heifers on the high plane of nutrition demonstrated a greater response to estrogen than heifers receiving the low plane of nutrition and response at 4 wk was significantly greater than at 2 wk postpartum. Similar results have been reported in crossbred heifers nutritionally restricted during the last two trimesters of gestation (Killen *et al.*, 1989). At 90 days of gestation, heifers received an experimental diet fed at 1, 1.5 or 2% of body weight to achieve body weight loss, maintenance or gain, respectively, at parturition. Heifers were injected with GnRH (100 $\mu$g, i.m.) between day 14 and 1 prior to or day 8 and 21 after parturition to evaluate the effects of nutrition on pituitary response to GnRH. Percentage change in bodyweight did differ among groups with a decrease in body weight reported for the loss (-17.6%) and maintenance (-6.0%) groups and an increase (+7.0%) for the gain group. Mean basal LH

secretion did not differ among groups either before or after parturition. Prepartum LH release was significantly greater (nearly twofold) in the body weight loss and maintenance groups compared with the body weight gain group and it was proposed that nutritional restriction during gestation may have suppressed endogenous GnRH secretion resulting in increased pituitary stores of LH. Conversely, postpartum response to GnRH was significantly greater (about 50%) in the body weight gain compared with the maintenance and loss groups and it was suggested that the detrimental effects of nutrient restriction on postpartum LH release might involve a reduction in pituitary content of LH. This latter suggestion is supported by the finding that energy intake significantly reduced mean serum LH concentrations on days 42 and 56, but not 28, postpartum in beef cows receiving a low (82% of the metabolizable energy requirement for a 375 kg cow with average to superior milking ability) compared with high (124% of the described energy requirement) energy diet beginning at calving (Hall *et al.*, 1991). Further, pituitary content of LH may not be restored as quickly in nutritionally restricted postpartum cows because, although mean LH concentration increased with increasing days postpartum in cows on the high energy diet no similar increase was observed in cows receiving the low energy diet.

Circumstantial evidence exists to support the supposition that dietary energy influences LH synthesis and secretion in postpartum cows through modulation of GnRH secretion. Postpartum beef cows received maintenance (100% NRC recommendations) or low (70% NRC) energy diets from day 190 of gestation until parturition followed by either low or high (130% NRC) energy diets after calving (Connor *et al.*, 1990). Content of GnRH in hypothalamus proper (HYP) and preoptic area (POA) and basal and $K^+$-induced release of GnRH from the stalk median eminence (SME) of day 30 postpartum cows was

determined. Neither pre- or postpartum diet altered the content of GnRH in the HYP or SME, however, content of GnRH in the POA was lower in cows on the high compared with low energy postpartum diet. Further, cows receiving the low energy diet both before and after parturition had a greater quantity of GnRH in the POA than those cows receiving the low energy diet prepartum followed by the high energy diet postpartum. These data suggest that GnRH release from the POA may be reduced in undernourished postpartum cows (resulting in increased tissue content of the decapeptide) and that the detrimental effects of underfeeding cows prior to calving, on subsequent GnRH secretion, may be overcome by feeding a diet high in energy after calving.

Although most of the nutritional studies investigating the relationship between diet and postpartum reproduction in beef cows have examined the effects of total dietary energy level on various reproductive parameters, several research groups have demonstrated that altering dietary crude protein level can also influence postpartum reproductive performance and LH secretion (Nolan *et al.*, 1988; Sasser *et al.*, 1988). In one experiment, first-calf beef cows were individually fed isocaloric diets, meeting 100% of NRC requirement for energy, that were either deficient (.32 kg/day) or adequate (.96 kg/day) in crude protein content (Sasser *et al.*, 1988). Diets were initiated 150 days prepartum and continued through day 40 postpartum at which time cows were group fed until the end of the experiment, day 110 postpartum. At parturition, total nutrient intake was increased 33% for all cows to meet lactational requirements. Cows were observed twice daily for estrus beginning day 12 postpartum and were artificially inseminated at each estrus from day 45 postpartum until the end of the experiment. Significantly fewer cows receiving the diet deficient in crude protein had returned to estrus and were determined to be pregnant after

the first service than cows receiving the diet adequate in crude protein. Further, although not significant statistically, cows receiving the crude protein deficient diet tended to have longer postpartum periods, intervals to first-service and a lower overall pregnancy rate. Collectively, these data demonstrate that limiting the intake of crude protein, but not total energy, prior to parturition is sufficient to negatively influence many aspects of postpartum reproduction in beef cows.

In a related series of experiments, the effects of restricting crude protein intake prior to calving on the hypothalamic-pituitary-ovarian axis was examined in postpartum beef cows (Nolan *et al.*, 1988). In experiments 1 and 2, Hereford cows were individually fed an isocaloric diet adequate (.96 kg/day) or deficient (.32 kg/day) in crude protein commencing 90, 60 or 30 days prior to parturition. After calving, feed intakes were increased (33%) to compensate for lactational demands. Cows, in experiment 1, were cannulated and blood samples collected at 15 min intervals for 8 hr on days 20, 40 and 60 postpartum were analyzed for LH. On day 62, pituitaries were collected and analyzed for GnRH receptor number and gonadotropin content. In experiment 2, pituitary response to exogenous GnRH (.22 $\mu$g/kg BW, i.m.) was investigated in cows on days 20, 40 and 60 after calving. Cows in experiment 3 received either the protein adequate or deficient diet 120 days prior to calving and pituitary response to estradiol (1 mg in safflower oil, i.m.) was evaluated on days 19, 39, and 59 after parturition.

Results from experiment 1 revealed that the pulse frequency, amplitude and basal and mean LH concentrations did not differ among the three protein restricted groups. Data from the three groups were combined and compared with those of cows receiving adequate levels of crude protein. Although no significant effect of crude protein on LH secretion on days

20, 40 or 60 was detected, a significant time effect on LH pulse frequency was observed. Cows fed the diet adequate in crude protein displayed an increase in LH pulse frequency with increasing days postpartum and by day 60 after calving LH pulse frequency was twice that of the cows receiving the diet deficient in crude protein. Numbers of receptors for GnRH in the anterior pituitary were not affected by diet. Pituitary content of LH was greatest in cows receiving the crude protein deficient diet starting 30 days prior to parturition and declined as length of crude protein restriction increased, finally reaching levels similar to those observed in cows receiving adequate levels of crude protein.

In the second experiment, both dietary crude protein and length of time after calving influenced pituitary response to GnRH. Amplitude of the GnRH-induced LH peak was greater in cows receiving an adequate crude protein level compared with all groups of protein-restricted cows. In addition, amplitude of the LH peak increased linearly with increasing days postpartum in cows maintained on a diet adequate, but not deficient, in crude protein. Dietary crude protein level also affected the time from GnRH injection to LH peak and the total amount of LH released in response to the decapeptide (area under the response curve). Cows on the restricted crude protein diet exhibited a shorter interval from injection to LH peak, however, total amount of LH released in response to GnRH was greater in cows receiving an adequate level of crude protein and the response increased with increasing days postpartum.

Response to exogenous estradiol, experiment 3, was variable in all cows and although there was no significant effect of dietary treatment on the number of cows responding to steroid, more cows receiving the diet adequate in crude protein responded with increased LH secretion compared with those on the restricted crude protein diet.

Collectively, the results of Sasser and coworkers (1988) and Nolan and colleagues (1988) clearly demonstrate that limiting crude protein intake prior to and after calving alters many characteristics of postpartum reproduction. Crude protein deficiency extends the postpartum interval, interval to first service and overall pregnancy rate in beef cows. In addition, the effects of crude protein deficiency appear to be mediated through alteration of the hypothalamic-pituitary axis, because feeding cows a protein restricted diet delayed the increase in LH pulse frequency, reduced pituitary response to exogenous GnRH and somewhat reduced hypothalamic and(or) pituitary sensitivity to exogenous estradiol after calving. It was suggested (Nolan *et al.*, 1988) that in crude protein restricted cows, the lack of recovery of pulsatile LH secretion after parturition might be due to reduced hypothalamic release of GnRH. Diminished LH response to exogenous GnRH, in the face of greater pituitary content of LH in dietary restricted cows, might result from a decrease in the releasable pool of gonadotropin and(or) a lack of functional GnRH receptors. Further, reduced response to estradiol might arise from decreased numbers of estradiol receptors in either the pituitary or hypothalamus.

Negative consequences of feeding a diet low in total dietary energy or crude protein to beef cows prior to and(or) after parturition appear to result from a delay in the recovery of a normal pattern of LH secretion after calving which likely impedes ovarian follicular development, ovulation and the return to regular estrous cycles. Further, the suppressive effects of restricted nutrient intake on postpartum LH secretion may be primarily mediated at the level of the hypothalamus through a reduction in the secretion of GnRH. Evidence suggesting that undernutrition reduces hypothalamic secretion of GnRH has also been reported to occur in cyclic beef cows (Rasby *et al.*, 1992). Content of GnRH in the

infundibular stalk-median eminence (ISME) was negatively correlated with body condition score ($r$=-.54) indicating that thin cows had more and fatter cows less GnRH in the ISME. These data were interpreted to suggest that GnRH release is reduced in thin undernourished cows.

**Suckling**

In many mammalian species, suckling during lactation results in an extended postpartum interval in which ovulation and the return to normal ovarian cycles is delayed. For the domestic livestock producer, the detrimental effect of suckling on postpartum rebreeding efforts results in reduced reproductive efficiency and subsequent financial loss. It is generally accepted that suckling negatively influences the neuroendocrine regulation of LH secretion after the hypothalamic-pituitary axis has recovered from the negative feedback of estradiol and progesterone during pregnancy and parturition. Suckling-mediated suppression of gonadotropin secretion in rats, farm animals, humans and non-human primates has been recently reviewed (McNeilly, 1988; Smith *et al.*, 1990; Williams, 1990).

Early research demonstrated that suckling delayed the return to estrus in beef cows independent of nutrient intake and that removal of the mammary gland (mastectomy) prior to calving, shortened the postpartum interval (Short *et al.*, 1972). More recently, it was reported that interaction of the calf with its dam suppressed ovulation and estrus independent of lactation and suckling (Viker *et al.*, 1989). In the latter experiment, mastectomized cows were allowed to remain with or were separated from their calves at birth. Calves remaining with their dams were hand fed from buckets or bottles and cows separated from their calves were in sight and sound of the other cows and calves but could not make contact with them.

Cows remaining with their calves did not ovulate or express estrus until calves were removed, days 46 to 53 postpartum. In contrast, all cows without calves had ovulated by day 22 (mean = day 16) and expressed estrus by day 40 (mean = day 24) postpartum. These data provided evidence that physical contact, other than suckling, with the calf could inhibit estrus and ovulation in mastectomized beef cows.

In a series of well-planned experiments, the effects of suckling on serum and follicular fluid hormones and follicular gonadotropin receptors (Walters *et al.*, 1982a), pituitary and ovarian function (Walters *et al.*, 1982b) and the induction of estrus, ovulation and luteal function (Walters *et al.*, 1982c) were thoroughly examined. Results from the first set of experiments (Walters *et al.*, 1982a) revealed that weaning calves from their dams on day 21 postpartum increased pulsatile LH secretion. In addition, prolactin content in follicular fluid and the number of follicular receptors for LH were increased in weaned cows and these two parameters were highly correlated ($r$=.85). In contrast, weaning had no effect on serum or follicular fluid levels of estrone, estradiol and progesterone or follicular fluid content of LH. These data suggested that the suppressive effects of suckling on the return to estrus and ovulation after calving result from reduced secretion of LH, and that this alone or in combination with reduced accumulation of prolactin in follicular fluid might suppress the numbers of follicular receptors for LH.

Evaluation of pituitary and ovarian function in suckled and weaned cows demonstrated that weaning calves on day 21 postpartum had no effect on pituitary weight or LH concentration but increased pituitary responsiveness to GnRH *in vitro* (Walters *et al.*, 1982b). Basal serum LH and FSH concentrations, content of prolactin in follicular fluid, and LH and FSH receptor numbers in the largest follicle were increased in weaned, but not

suckled, cows. Similar increases were observed just prior to ovulation in cycling beef heifers, in which estrus was synchronized using progestogen ear implants. In addition to reducing pituitary responsiveness to GnRH, the suckling stimulus was suggested to reduce the frequency of GnRH pulses from the hypothalamus because removal of progestogen implants from cyclic cows resulted in endocrine changes very similar to those observed in untreated weaned cows. Further, the inhibitory effect of progesterone on LH secretion is thought to be mediated by a reduction in the frequency of GnRH release (Goodman and Karsch, 1980). Similar results were reported by Parfet and coworkers (1986) in which it was concluded that suckling inhibited neural elements responsible for the synthesis and(or) secretion of GnRH, thus, suppressing the secretion of LH and delaying the return to estrus after calving.

The hypothesis that suckling prolongs the postpartum interval by reducing the frequency of GnRH and subsequently LH secretion was indirectly tested by injecting suckled beef cows with small doses of GnRH, to produce a pattern of LH secretion similar to that observed in weaned cows, in an attempt to induce estrus and ovulation (Walters *et al.*, 1982c). Further, because estradiol can influence pituitary responsiveness to GnRH, the effect of exogenous estradiol on the LH response to GnRH injections was also evaluated. Injections of GnRH (500 ng), in the presence or absence of exogenous estradiol implants, were administered to suckled cows every 2 hr for 4 days beginning on days 18 to 22 postpartum. Cows receiving intermittent injections of GnRH had a greater number of LH pulses that were of greater magnitude than basal concentrations of the gonadotropin in suckled control cows and suckled cows receiving estradiol alone. Suckled cows receiving estradiol alone had fewer LH pulses than suckled control cows and cows receiving GnRH

and GnRH+estradiol. Duration of the postpartum interval was reduced in weaned cows and in suckled cows treated with GnRH or GnRH+estradiol compared with suckled control cows and cows receiving estradiol alone. In fact, suckled cows receiving only estradiol had postpartum intervals that were, on average, 10 days longer than those of suckled control cows, suggesting that exogenous estradiol may delay the return to estrus and ovulation by reducing LH secretion. Estradiol failed to reduce LH pulse frequency or increase the postpartum interval in suckled cows receiving GnRH injections. Collectively, the results of this experiment support the concept that suckling may prolong the postpartum interval by reducing the frequency of GnRH, and subsequently, LH pulses.

Initial studies, to determine the mechanism underlying suckling-induced suppression of estrus and ovulation after parturition in cows, focused on the role of ovarian steroids in the modulation of hypothalamic-pituitary function. Prior research indicated an increased negative feedback effect of estradiol on LH secretion after lambing in suckled OVX ewes compared with OVX cyclic ewes (Wright *et al.*, 1981). Similar results have been observed in ovariectomized postpartum cows treated with estrogen (Acosta *et al.*, 1983). Estradiol suppressed the frequency of LH release in cows nursing a calf compared with similarly treated cows subjected to early weaning. From these data, Acosta and coworkers proposed that suckling prolongs the postpartum interval by increasing the sensitivity of the hypothalamus to the negative feedback of estrogen thus reducing LH release from the pituitary.

It has also been proposed that the suckling stimulus alters hypothalamic-pituitary response to low levels of progesterone in postpartum cows (Williams *et al.*, 1983). In this study, suckled and nonsuckled postpartum cows received none or two progesterone implants

on day 7 after calving. On day 14, blood was collected at 10 min intervals for 6 hr and analyzed for LH. Progesterone implants chronically increased systemic levels of the steroid (.5 to .6 ng/ml) in treated cows and increased mean plasma levels of LH, as well as, LH pulse frequency in nonsuckled, but not suckled, cows. Further, progesterone appeared to potentiate the positive effects of weaning on LH secretion because, although LH secretion was greater in nonsuckled nonimplanted cows than in suckled nonimplanted cows, the effect was even more pronounced when nonsuckled cows were exposed to low levels of the steroid for 1 wk. In addition, because exposure to low levels of progesterone after calving is thought to aid in the initiation of regular estrous cycles (LaVoie *et al.*, 1981), Williams and coworkers (1983) suggested that the suppressive effect of suckling on LH secretion may, in part, result from a failure of the hypothalamic-pituitary axis to respond to the putative stimulatory effects of this steroid.

A growing body of evidence, that endogenous opioid peptides negatively regulated pulsatile LH secretion during the estrous cycle of many species (for review, see Kalra, 1986; Barb *et al.*, 1991), sparked subsequent research to determine if these peptides played a similar role in regulating LH release during the postpartum period. Initial experiments using postpartum cows (Gregg *et al.*, 1986; Whisnant *et al.*, 1986b,c), ewes (Gregg *et al.*, 1986; Malven and Hudgens, 1987) and sows (Barb *et al.*, 1986; Mattioli *et al.*, 1986) characterized LH response to naloxone, an opioid antagonist. Collectively, results from these experiments suggested that endogenous opioid peptides were involved in the control of LH secretion after parturition in domestic livestock because administration of naloxone increased systemic concentrations of the gonadotropin. Additional support was provided by the results of an experiment that evaluated the effect of morphine, a nonselective opioid

agonist, on gonadotropin secretion in weaned postpartum beef cows (Peck *et al.*, 1988). Morphine injection (1 mg/kg body weight) decreased LH pulse frequency during the 5 hr bleeding period but only decreased mean serum LH concentration from 105 to 270 min after treatment. Infusion of the opiate (.31 mg/kg body weight followed by .15 mg/kg per hr for 7 hr) decreased LH pulse frequency and LH response to exogenous GnRH, but was without effect on mean serum LH concentration..

Initially, it was proposed that brain opioid tone in postpartum beef cows decreased (less opioid antagonist required to elicit LH response) with increasing days after calving because suckled cows failed to respond to naloxone on days 14 and 28, but not day 42, postpartum (Whisnant *et al.*, 1986c) and a larger dose of the antagonist was required to increase systemic LH concentrations in cows on day 14 than on days 28 and 42 (Whisnant *et al.*, 1986b). However, Barb and coworkers (1991) suggested that the lack of response to naloxone challenge prior to day 30 postpartum may also be explained, in part, by a reduction in pituitary response to GnRH.

Because both calf removal and administration of naloxone resulted in enhanced LH secretion, Whisnant and coworkers (1986a) conducted an experiment to evaluate the effect of calf removal on naloxone-induced LH secretion in beef cows. Removal of calves for 48 hr increased serum LH concentrations prior to, but not after, naloxone (200 mg) treatment. In contrast, suckled control cows had low serum concentrations of LH prior to naloxone injection and responded to the antagonist with increased LH release. These data provided indirect evidence that endogenous opioid peptides might be involved in the suckling-induced suppression of LH release in postpartum beef cows.

Further support for opioid involvement in the suckling-mediated suppression of LH secretion was provided by Malven and coworkers (1986). Concentrations of dynorphin A (amino acids 1-17), methionine (met)-enkephalin and GnRH increased in the preoptic area (POA) and hypothalamus of postpartum beef cows between 36 and 72 hr after calf removal. It was suggested that the observed increase might arise from decreased secretion and(or) turnover of the opioid peptides, either of which would reduce opioid-mediated inhibition of GnRH synthesis and(or) secretion. Although results of a subsequent study (Connor *et al.*, 1990) were not in complete agreement with those described above, both experiments demonstrated time-dependent changes in the concentration of $\beta$-endorphin, met-enkephalin, dynorphin A and GnRH in POA and hypothalamic tissues following calf removal.

More recently, Leshin and coworkers (1992b) reported the distribution of immunoreactive (ir) $\beta$-endorphin neurons in the arcuate nucleus and median eminence of suckled anestrous beef cows during the early (days 10 to 16) and middle (days 33 to 43) stages of the postpartum interval and cyclic (12 to 14 months postpartum) beef cows during the luteal phase. In all cows, ir-$\beta$-endorphin perikarya were primarily located in the arcuate and periarcuate nucleus with some located in the dorsolateral and posterior aspects of the median eminence. Percentage area of ir-$\beta$-endorphin within the arcuate nucleus was significantly greater in cyclic ($31 \pm 5.6\%$) than in early postpartum ($16.1 \pm 1.7\%$) cows. In mid-postpartum cows, the percentage area of immunostaining ($20.6 \pm 3.2\%$) was intermediate to, but not significantly different, from the other groups. Total area measured did not differ between groups. In contrast, the percentage area of ir-$\beta$-endorphin in the median eminence was similar for all groups. These data support the hypothesis that endogenous opioid peptides are involved in mediating the suppressive effects of suckling on LH secretion and

because extensive intermingling of ir-$\beta$-endorphin and GnRH varicosities was commonly observed in the external lamina of the median eminence in all cows, the authors suggested that $\beta$-endorphin may suppress the activity of GnRH neurons in early postpartum cows.

Opioid binding sites for naloxone have been quantified in hypothalamus, POA and basal forebrain (BF) tissues collected from suckled beef cows on day 7, 14, 28, 42 or 56 after calving (Trout and Malven, 1988). All cows slaughtered on days 7, 14 and 28 were still anestrus; however, a majority of cows sacrificed on day 42 (5 of 8) and 56 (5 of 6) had resumed cycling. Anestrous cows had a greater number of naloxone binding sites in POA and BF tissues than did cyclic cows. Additionally, the concentration of binding sites was greater in BF than in POA or hypothalamic tissues, similar to that reported for ewes (Weesner *et al.*, 1989). From these data, it was suggested that the observed decrease in the number of naloxone binding sites, in the POA and BF of cyclic cows, might be due to progesterone feedback and could reflect a reduction in the ability of endogenous opioid peptides to inhibit pulsatile LH secretion.

Effect of progesterone on LH response to naloxone in postpartum beef cows has been investigated (Cross *et al.*, 1987). Cows were untreated or received twice daily injections of progesterone (50 mg, i.m.) from days 16 to 19 postpartum. On days 18 and 19, each cow received one injection of naloxone (275 mg, i.v. at -.5 hr and 4 hr later) and blood was collected at 10 min intervals for 8 (day 18) or 4 (day 19) hr beginning 30 min after the first injection. Cows receiving the steroid treatment had serum progesterone levels comparable to those observed during the luteal phase (3.8 $\pm$ .44 ng/ml), whereas, serum progesterone levels in untreated cows averaged less than 1 ng/ml. Cows treated with progesterone for 4 days failed to respond to the naloxone injections, suggesting a reduction in the inhibitory

influence of opioids. In contrast, cows not receiving the steroid treatment had significantly greater mean serum concentration, pulse amplitude, pulse duration and pulse frequency of LH in response to naloxone. Further, LH data from steroid-treated cows were not significantly different from those of untreated control cows injected with saline instead of naloxone. Data from this experiment support a role for progesterone in the suppression of opioid inhibition of LH secretion in postpartum cows and, interestingly, administration of the opioid antagonist quadazocine (WIN 44441-3) to luteal phase heifers also failed to stimulate LH secretion (Short *et al.*, 1987).

Opioid inhibition of LH secretion appears to be, at least in part, a steroid-dependent phenomenon because LH secretion was unaffected in long-term OVX ewes treated with opioid antagonist and inhibition could be restored upon treatment with ovarian steroids (Brooks *et al.*, 1986b; Whisnant and Goodman, 1988). This also appears to be the case for the cow, because naloxone failed to stimulate LH secretion in late pregnant and steroid-treated OVX beef cows (Rund *et al.*, 1990). In addition, after steroid withdrawal serum LH concentrations were increased by naloxone treatment suggesting that the effects of progesterone and estradiol were mediated through an opioid-dependent mechanism.

More recently, administration of naloxone (1 mg/kg body weight, i.v.) failed to increase systemic levels of LH in suckled and nonsuckled OVX (6 months) postpartum beef cows, suggesting an absence of opioid inhibition (Rund *et al.*, 1992). Lack of opioid inhibition most likely occurred from the chronic absence of ovarian steroids due to long-term ovariectomy, but also may reflect a decrease in the opioid-dependent effects of suckling on LH secretion this late in the postpartum interval ($191 \pm 7$ days). Interestingly, LH secretion was inhibited in both suckled and nonsuckled cows after administration of morphine

(1 mg/kg body weight, i.v.) suggesting that opioid receptors are present late in the postpartum interval of long-term OVX cows and that binding of a nonselective opioid agonist can inhibit endogenous GnRH release and(or) reduce pituitary sensitivity to the decapeptide. Response to a suboptimal dose of exogenous GnRH (5 $\mu$g, i.v.) was greater in nonsuckled than in suckled cows suggesting a direct effect of suckling on pituitary responsiveness to GnRH stimulation. The mechanism through which suckling suppressed pituitary responsiveness to GnRH is not currently known; however, some circumstantial evidence exists to support a role for a direct action of endogenous opioids on the pituitary. Hypothalamic portal blood concentrations of $\beta$-endorphin are increased during suckling in postpartum ewes (Gordon *et al.*, 1987) and met-enkephalin inhibited GnRH-induced LH release from bovine pituitary cells *in vitro* (Chao *et al.*, 1986). From these data, Rund and coworkers (1992) have suggested that, in long-term OVX postpartum cows, suckling may increase pituitary content of opioid peptides which in turn may modulate pituitary response to GnRH. Alternatively, it was suggested that the differential response to GnRH in suckled and nonsuckled cows might result from differences in the quantity of releasable pools of LH.

Presumably, if suckling reduced pituitary responsiveness to GnRH in long-term OVX postpartum cows, one would anticipate a difference in LH secretion in response to endogenous GnRH stimulation. However, in this study pretreatment mean LH concentration, and pulse amplitude and frequency did not differ between suckled and nonsuckled cows, suggesting that this is not the case. Therefore, it appears more likely that the quantity of releasable pools of LH might be responsible for the suppressed response to exogenous GnRH in suckled cows. Additionally, because secretion of LH did not differ

between the two groups prior to GnRH treatment, the difference in releasable pools of LH, in this case, may not be of biological importance.

Clearly, the mechanism through which endogenous opioid peptides mediate the suckling-induced suppression of LH secretion in postpartum cows is complex and likely involves regulation at both the hypothalamus and pituitary. Further, this mechanism appears to be dependent on the presence of ovarian steroids, however, the precise mechanism, and site(s) of action, whereby progesterone and(or) estrogen act to modulate opioid responsiveness in suckled cows has yet to be determined.

**Season**

Unlike other domestic ruminants, such as sheep and goats, reproductive activity and ovarian cycles are not restricted to a specific season of the year in cows. Although successful breeding of cyclic cows is not dependent on prevailing daylength, photoperiod has been shown to influence age of attainment of puberty (Schillo *et al.*, 1983), ovarian activity (McNatty *et al.*, 1984c; Mascarenhas *et al.*, 1986) and duration of the postpartum interval (Hansen and Hauser, 1983; King and Macleod, 1984) suggesting that some aspects of reproduction in this species are responsive to seasonal daylength cues. Seasonal reproduction in domestic farm species has been reviewed (Ortavant *et al.*, 1985).

Critser and coworkers (1983) provided evidence to support the concept that, in the bovine, there is an underlying reproductive mechanism that is responsive to changes in season. Mean systemic concentrations of LH in OVX dairy heifers were significantly greater during the winter than during the summer solstice periods. Maximal levels of the gonadotropin were observed from November to April and minimal levels from May to

October, thus demonstrating an inherent distinct pattern of LH release that was dependent upon the prevailing photoperiod. In a second experiment, similar LH profiles were obtained when OVX dairy heifers were treated with estradiol implants, except that overall systemic LH concentrations were elevated in heifers receiving the steroid compared with untreated control heifers. Day and coworkers (1986) confirmed that photoperiod influences bovine gonadotropin secretion, and further reported that the maximal and minimal concentrations of LH observed in OVX mature cows at the time of the fall and spring equinoxes, respectively, resulted from cyclical alterations in the amplitude, but not frequency of LH pulses.

In contrast to the results of these studies, untreated OVX ewes exhibit high mean systemic levels of LH throughout the year, and long-term estrogen treatment abrogates LH secretion during the non-breeding season, similar to that observed in ovary intact ewes (Legan *et al.*, 1977; Legan and Karsch, 1980). It should be noted, however, that although mean LH levels were unaffected by season, differences in LH pulse frequency and amplitude were observed in untreated OVX ewes during the breeding season and anestrus (Goodman *et al*, 1982) demonstrating a direct effect of photoperiod on LH secretion in the absence of gonadal steroids. Therefore, the influence of season on basal LH secretion in the OVX cows and heifers is not markedly altered in the presence estradiol, whereas in the ewe, a definite seasonal breeder, seasonal variations in LH pulse frequency and amplitude that occur in the absence of gonadal steroids are profoundly enhanced in the presence of estradiol.

Photoperiod has also been shown to alter systemic concentrations of LH in cyclic Brahman cows (Harrison *et al.*, 1982), Angus cows (McNatty *et al.*, 1984c) and in ovariectomized beef heifers (Critser *et al.*, 1987b). Harrison and coworkers (1982) reported

that, in Brahman cows, levels of LH during the preovulatory gonadotropin surge were significantly greater during early (March) and late (May) spring than during the winter (early January). Contrary to this report, exposure of cyclic dairy heifers to photoperiods of 8 hr of light and 16 hr of dark (8L:16D) or 16L:8D did not alter the timing, pattern or amplitude of the preovulatory LH surge (Rzepkowski *et al.*, 1982), however, the heifers in this study were exposed to a constant photoperiod whereas the cows in the former experiment (Harrison *et al.*, 1982) were exposed to a changing photoperiod and perhaps the gradual change in daylength that occurs under natural conditions, rather than absolute hours of light and dark, is required to alter LH secretion in cattle.

In cyclic beef cows, fluctuations in LH pulse frequency during proestrus have also been attributed to changes in the season (McNatty *et al.*, 1984c). A greater number of LH pulses, between -5 and -1 days of the estrous cycle (day 0 = day of estrus), occurred in cows during the spring than during the autumn and winter. Conversely, corpora lutea (CL) were heavier and secreted more progesterone in the autumn and winter than in the spring. From these data, the reduction in LH pulse frequency observed in fall and winter was suggested to result from progesterone negative feedback because mean plasma progesterone levels were greater at this time of the cycle in cows during the autumn and winter. The inhibitory effect of progesterone on LH pulse frequency has been well documented in ewes (Goodman *et al.*, 1981).

Further support for an underlying effect of photoperiod on LH secretion in the bovine was provided by Critser and colleagues (1987b). These investigators examined the effect of seasonal transitions on LH secretion in estradiol-treated ovariectomized beef heifers. Exposure of heifers to the natural photoperiod of fall to spring (September to March)

increased basal concentration and pulse amplitude, but not pulse frequency or duration, of LH compared with heifers exposed to supplemental lighting to simulate the photoperiod of spring to fall (vernal equinox to autumnal equinox).

Season has been demonstrated to influence the interval from calving to first estrus in postpartum beef cows (Peters and Riley, 1982). Cows calving between November and April had significantly longer postpartum intervals (70.8±2.6 days) than cows calving from May through October (35.9±2.6 days). Further, although there was no correlation between photoperiod on the day of calving and the length of the postpartum interval, daily photoperiod 1 month prior to calving was negatively correlated ($r$ = -.737; $P < .001$) with the duration of postpartum anestrus suggesting that cows exposed to longer photoperiod (May to October) late in gestation return to regular ovarian cycles earlier than do those cows exposed to shorter photoperiod (November to April). From these data, it was suggested that, in the cow, a vestigial sensitivity to photoperiod is present and is similar to that observed in long-day breeders, such as the mare.

Similar results were reported by Hansen and Hauser (1983) and King and Macleod (1984) in which postpartum beef cows calving in the summer and fall had shorter postpartum intervals than those calving in the winter or spring. Further, exposure of autumn and winter calving beef cows to a photoperiod of 18L:6D significantly reduced the length of the postpartum interval, but failed to alter LH secretion, compared with cows exposed to the natural photoperiod (Hansen and Hauser, 1984). It was proposed that calving would occur more frequently in the spring and summer than in winter, if cows were bred at each estrus (Hansen and Hauser, 1983), supporting the hypothesis that cows may intrinsically follow the seasonal reproductive pattern of a long-day breeder, as suggested by Peters and Riley

(1982). This view is in apparent disagreement with that advanced by Critser and coworkers (1983) in which the underlying seasonality of LH secretion in the cow was proposed to be more closely related to the evident seasonality of reproduction in the ewe (a short-day breeder) and not the mare, because LH profiles in OVX heifers more closely approximated those of estrogen-treated OVX ewes but not OVX mares.

Collectively, results from these experiments support the concept that, in the cow, LH secretion and length of the postpartum interval may be modulated by the changing photoperiod. Interestingly, cyclic cows exhibited enhanced LH secretion in the spring (Harrison *et al.*, 1982; McNatty *et al.*, 1984c), whereas, secretion of this gonadotropin in estradiol-treated ovariectomized heifers was greater in the winter and from fall to spring (Critser *et al.*, 1983; Critser *et al.*, 1987b). It is difficult to reconcile the differences in LH data obtained from experiments with cyclic cows and ovariectomized heifers because in most instances the duration of exposure to various photoperiods was markedly different and, although the intrinsic seasonal profile of LH secretion observed in OVX heifers was not profoundly altered by exposure to estradiol, it is possible that progesterone alone or in combination with estradiol may alter the seasonal LH profile in these heifers to more closely resemble that of ovary intact cows.

Data from experiments conducted to determine the effect of calving season on the interval from parturition to first estrus appear to be more consistent than those concerning the effect of season on LH secretion. There is general agreement that the postpartum interval is shorter in cows calving in the summer and fall or under an artificial long day photoperiod (18L:6D) compared with those calving in the winter or spring (Peters and Riley, 1982; Hansen and Hauser, 1983; Hansen and Hauser, 1984; King and Macleod, 1984).

Although, the mechanism underlying seasonal modulation of the postpartum interval is presently unknown, it seems very likely that changes in photoperiod, associated with the passing of seasons, provoke an alteration in GnRH pulse generator activity and subsequent LH release, as has been postulated to occur during the initiation and termination of the breeding season in the ewe (for review, see Karsch *et al.*, 1984; Karsch and Woodfill, 1992).

Melatonin, secreted by the pineal gland, is believed to mediate neuroendocrine responses to seasonal changes in daylength. The action of this indoleamine on the hypothalamic-pituitary axis, to regulate the secretion of LH, has been particularly well-studied in the ewe. However, although a number studies have demonstrated seasonally-induced alterations in LH secretion or the postpartum interval in cows, few experiments have been conducted to determine if these photoperiod-dependent changes are mediated through or can be induced by melatonin. In one such experiment, Sharpe and coworkers (1986) investigated the effect of melatonin on the duration of the postpartum interval in Shorthorn cows. Autumn-calving cows (day 4 to 38 postpartum) receiving melatonin (500 mg in beef tallow) had significantly higher daytime concentrations of melatonin and longer intervals to first estrus ($68\pm4$ vs $58\pm5$ days) and ovulation ($68\pm4$ vs $55\pm5$) than untreated control cows, suggesting that melatonin may mediate the effects of photoperiod on the return to estrus and ovulation in this species. Unfortunately, because the cows were not sampled for LH at any time during the experiment, the effect of the indole on gonadotropin secretion could not be determined. Interestingly, if the extended daily exposure to elevated concentrations of melatonin (exogenous plus endogenous nocturnal rise) was perceived as a short-day photoperiod similar to that of winter, then these results would be consistent with

the finding that fall-calving cows have shorter postpartum intervals than cows calving in winter and spring.

# Role of Melatonin in the Regulation of Reproduction in Sheep and Cows

It is now well accepted that the mammalian pineal gland, through secretion of melatonin, is involved in the synchronization of specific physiological processes (*e.g.* feeding and social behavior, activity, pelage growth and reproductive events) to daily and seasonal changes in the external environment (Binkley, 1988). Because melatonin is secreted during the scotophase (dark period) and the duration of nocturnal elevation is proportional to the hours of darkness, this indoleamine provides information on daylength (photoperiod), in the form of a hormonal signal (for review, see Bartness and Goldman, 1989; Cassone, 1990; Reiter, 1991a). With respect to the seasonal reproductive cycles of certain domestic ruminants and other species, the circadian pattern of melatonin release acts to synchronize the breeding season to a specific time of the year such that parturition occurs when environmental conditions are most favorable for growth and survival of the young (Karsch *et al.*, 1984).

Of the livestock species considered to be seasonal breeders, photoperiodic regulation of circannual reproductive cycles has been most extensively studied in the ewe; however, more recent research has included does (deer and goats) and to a lesser extent, mares. In the case of the cow, the process of domestication appears to have resulted in the progressive selection of animals that were capable of breeding throughout the year. However, although bovine reproductive activity is not restricted to a specific season of the year, a vestigial sensitivity to change in duration of daylight appears to have been retained, because photoperiod has been demonstrated to influence growth, attainment of puberty, and as discussed previously, ovarian function and the return to estrus after calving. Further,

because cows are not considered to be seasonal breeders, research investigating the physiological basis underlying the observed effects of photoperiod on reproductive phenomena has not been pursued as vigorously as that for the ewe, resulting in a paucity of information concerning the functional role of melatonin in the control of reproduction in this species. The mechanism whereby the pineal gland regulates reproductive development and subsequent annual breeding seasons of domestic ruminants has been recently reviewed (Karsch *et al.*, 1991; Deveson *et al.*, 1992; Yellon *et al.*, 1992).

**The Pineal Gland**

The mammalian pineal gland originates embryonically as an evagination of ependymal cells that line the roof of the third ventricle. The gland extends from the area between the habenular and posterior commissures of the diencephalon and its size, shape and precise anatomical location vary among species (for review, see Reiter, 1981). Blood supply to the pineal is profuse, originating from branches of the posterior choroidal arteries, and numerous capillaries exist among the pinealocytes, the main cell type of this gland. In the rat, the blood supply to the pineal gland (volume per gram of tissue) was determined to be greater than that for any other endocrine gland with exception of the kidney (Goldman and Wurtman, 1964). Synthesis and release of pineal secretory products is controlled by postganglionic sympathetic neurons that originate in the superior cervical ganglia (SCG) and terminate primarily in pericapillary spaces within the gland. As a result, sympathetic innervation is requisite for melatonin synthesis and, thus, chemical expression of the light-dark cycle (for references, see Reiter, 1981). The mammalian pineal also receives central innervation from the habenula posterior commissure complex (Korf and Møller, 1984) and

there is evidence demonstrating neuropeptide Y (NPY) innervation of the ovine pineal; however, the role of NPY in pineal function is unclear because the peptide had no effect on adrenergic stimulation of melatonin synthesis (Williams *et al.*, 1989). Additionally, it has been suggested that the bovine pineal may receive cholinergic innervation (Phansuwan-Pujito *et al.*, 1990) and that muscarinic cholinergic fibers may play a role in modulating the synthesis of melatonin in this species (Pujito *et al.*, 1991).

The pineal gland of most mammalian species, including the cow and ewe, is mainly composed of two types of cells: **pinealocytes**, derived from photoreceptor cells, and **glial** or glial-like cells of neural origin (for review, see Pévet, 1981). The predominant cell type of pineal tissue is the pinealocyte, which contains the requisite enzymes for melatonin biosynthesis and is characterized by the presence of granular vesicles. In some species, two types of pinealocytes, light and dark, have been reported; however, the only observable difference between the two cell types was the degree of shading, and the functional significance of this finding is unclear. Glial cells reside among pinealocytes and, although their function has not been completely elucidated, it has been suggested that they provide support for the pinealocytes. Gross and microscopic analysis of the bovine and ovine pineal gland has been reported (Anderson, 1965).

***Synthesis and Secretion of Melatonin***

Over 30 years ago, melatonin was first isolated from bovine pineal glands and its chemical structure identified as N-acetyl-5-methoxytryptamine (Lerner, 1959). Since its discovery, an incredible wealth of information has accumulated regarding the pathway and regulatory sites of melatonin biosynthesis (for review, see Cardinali, 1981; Reiter, 1981; Sugden, 1989; Krause and Dubocovich, 1990; Reiter, 1991b).

The bulk of melatonin biosynthesis is restricted to the pineal, and to a lesser extent the retina, Harderian gland (Reiter *et al.*, 1983) and gastrointestinal tract (Lee *et al.*, 1991), by limited tissue distribution of two key enzymes, serotonin N-acetyltransferase (SNAT) and hydroxyindole-O-methyltransferase (HIOMT), which are requisite for the nocturnal production of this indolamine. Melatonin is derived from the amino acid tryptophan which appears to be actively transported, against a concentration gradient, from the systemic circulation into the pinealocyte (Sugden, 1979). In the first step of melatonin synthesis, tryptophan is converted to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase which transfers a hydroxy group to the 5-position of the indole ring. This reaction is thought to be the rate limiting step in the synthesis of serotonin (5-hydroxytryptamine; 5-HT), the precursor of melatonin (Bloom and Giarman, 1967).

Serotonin is produced from 5-hydroxytryptophan, through decarboxylation of the side-chain, by the enzyme 5-HTP decarboxylase. The concentration of serotonin in the pineal gland of most species greatly exceeds that found in virtually all regions of the brain (Quay, 1963) and other body tissues (Saavedra *et al.*, 1973). Pineal content of serotonin displays a diurnal rhythm in which levels are maximal during daylight hours and decrease after darkness concomitant with the nighttime rise in melatonin synthesis (for references, see Reiter, 1992b). More recently it has been suggested that, in addition to its role as the precursor of melatonin, serotonin may also assume an endocrine or paracrine role in modulation of bovine pineal function because stimulation of pinealocytes *in vitro* with norepinephrine or dopamine provoked the release of serotonin (Chuluyan *et al.*, 1989) and serotonin $5HT_2$ receptors have been recently identified ($K_d = 1.26 \times 10^{-9}$ M; $B_{max} = 193 \pm 38.85$ fmol/mg protein) in bovine pineal membranes (Govitrapong *et al.*, 1991).

Synthesis of melatonin from serotonin is a two-step process that begins with the N-acetylation of serotonin by the cytosolic enzyme SNAT to form N-acetylserotonin (NAS). This is generally accepted to be the rate limiting step in the biosynthesis of melatonin. Serotonin N-acetyltransferase has been purified from ovine (Namboodiri *et al.*, 1987a) and rat pineals (Namboodiri *et al.*, 1987b) and is believed to have a molecular weight of approximately 10 kDa; however, the amino acid sequence of the enzyme still remains to be determined. In the rat, SNAT activity is increased 30- to 70-fold upon darkness and is thought to contribute greatly to the nighttime rise in the synthesis and release of melatonin in this species (Klein *et al.*, 1981). In contrast, daytime SNAT activity in the ovine pineal is 25-fold higher than that of the rat and exhibits only a two to fourfold increase upon onset of darkness (Namboodiri *et al.*, 1985). Interestingly, despite the considerable difference in SNAT activity, daytime and nighttime levels of melatonin in sheep are similar to those reported for the rat, suggesting that the regulation of this key enzyme may differ between the two species. Currently there are no data concerning the daytime and nighttime activities of bovine pineal SNAT, but presumably the diurnal rhythm would be more similar to that reported for ovine, as compared to rat pineal glands.

The final step in melatonin synthesis (N-acetylserotonin → melatonin) is catalyzed by HIOMT, which resides in the cytosol of the pinealocyte. This enzyme transfers a methyl group from S-adenosylmethionine to the hydroxyl group of NAS. Purification of HIOMT from rat (Jackson and Lovenberg, 1971) and bovine (Karahasanoglu and Ozand, 1972) pineal revealed that the enzyme consists of two 38 kDa subunits. In addition, the nucleotide sequence of a cDNA encoding the bovine enzyme was reported (Ishida *et al.*, 1987) and subsequently revised (Donohoe *et al.*, 1992). Although early

HIOMT activity in rat pineal glands exhibited a diurnal rhythm, subsequent experiments using saturating concentrations of substrate failed to detect any cyclic pattern in activity (Sugden, 1989). Similarly, in sheep, nighttime activity of HIOMT does not differ from that observed during the day (Sugden *et al.*, 1985). The preferred substrate for HIOMT is NAS, however, several other indoles can be methylated by the enzyme (Axelrod and Weisbach, 1961). Further, because no storage or exocytotic mechanisms have been observed in mammalian pinealocytes, it is generally accepted that after synthesis melatonin is released from the pinealocyte by passive diffusion. Additionally, systemic concentrations of melatonin are believed to directly reflect pineal content of the indoleamine (Reiter, 1981; Cardinali, 1981; Sugden, 1989).

The daily pattern of melatonin production, in mammals, is thought to be generated by an endogenous circadian clock, located in the suprachiasmatic nuclei (SCN) of the hypothalamus, and is entrained to a 24 hr period by the light-dark cycle (Krause and Dubocovich, 1990; Reiter, 1991a,b; Yellon *et al.*, 1992). The diurnal pattern of melatonin synthesis persists, with a period slightly greater than 24 hr, in both sheep (Rollag and Niswender, 1976) and hamsters (Yellon *et al.*, 1982) under conditions of constant darkness but is abolished by exposure to constant light (Namboodiri *et al.*, 1985). Photic information is relayed to the pineal gland *via* a multisynaptic pathway. Light, striking photoreceptors in the retina, initiates the signal which is transmitted *via* the retinohypothalamic tract (RHT) to the SCN. From the SCN, the signal is conveyed as follows: SCN → paraventricular nuclei (PVN) → medial forebrain bundle → mediolateral nuclei of upper thoracic spinal cord → superior cervical ganglion (SCG) → sympathetic nerves (nervi conarii) → pinealocyte (for references, see Krause and Dubocovich, 1990).

Norepinephrine is the principal neurotransmitter within the sympathetic nerve terminals in the pineal gland. Release of the catecholamine is stimulated by darkness, and in the rat, but not other species, norepinephrine content in pineal postganglionic nerve terminals increases at night. The mechanism whereby norepinephrine stimulates pinealocyte melatonin synthesis has been extensively studied in the rat and other rodents (for review, see Sugden, 1989; Reiter, 1991b); however, over the past 5 years, this line of research has been extended to include sheep (for review, see Namboodiri *et al.*, 1991) and, to a lesser extent, cows.

It is important to note, that although melatonin is the primary compound synthesized by the mammalian pineal, a number of different indolic tryptophan derivatives and several peptides have also been identified in this tissue (for review, see Preslock, 1984). A sample of these minor pineal indoles includes 5-hydroxytryptamine, N-acetyl-5-hydroxytryptamine, 5-methoxytryptophol, 5-O-acetyl-5-methoxytryptophol and 5-methoxyindole-3-acetic acid. Despite evidence demonstrating that 5-methoxytryptamine can alter gonadal function in golden hamsters (Rollag, 1982), the biological relevance of this and other minor pineal indoles has yet to be determined for this and other species. Further, peptides isolated from pineal tissue of cows, and other species, include arginine vasotocin, oxytocin, arginine vasopressin, threonyl-seryl-lysine and luteinizing hormone-releasing hormone (LHRH), however, it has been suggested that these peptides may be accumulated, rather than synthesized, by pineal cells and once again the biological significance of their presence is unknown (Preslock, 1984).

Regulation of Serotonin N-acetyltransferase Activity

In the case of the rat, norepinephrine mediates its stimulatory effects on melatonin synthesis by binding to postsynaptic adrenergic receptors located in the pinealocyte cell membrane (for references, see Sugden, 1989; Reiter, 1991b). Approximately 85 percent of the nocturnal rise in melatonin in this species is attributed to the binding of norepinephrine to $\beta_1$-adrenoceptors. The remainder is presumed to arise from the interaction of the catecholamine with $\alpha_1$-adrenoceptors to potentiate the effect of $\beta$-adrenergic stimulation. Binding of norepinephrine to $\beta_1$-adrenoceptors provokes the synthesis of cAMP through activation of adenylyl cyclase by a guanine nucleotide binding protein, $G_s$, as has been delineated for the activation of the LH receptor in a previous chapter of this review. Elevated nighttime levels of pinealocyte cAMP are thought to activate cAMP-dependent protein kinase which in turn stimulates the induction of SNAT. The mechanism underlying the increase in SNAT activity is not well understood, however, cAMP-induced transcription of mRNA is requisite for increased N-acetylation of serotonin. To date, it is still unclear if the newly transcribed mRNA invokes *de novo* synthesis of the SNAT enzyme or a putative SNAT activator.

Recently, it was postulated that the nuclear phosphoprotein Fos, encoded by the proto-oncogene c-*fos* and thought to act as a transcriptional regulatory protein, may play a role in the control of melatonin synthesis by modulating serotonin metabolism. In support of this premise, Fos immunoreactivity (Koistinaho and Yang, 1990) and mRNA transcription (Carter, 1990) are increased during the dark period, and the induction of c-*fos* mRNA closely parallels the rise in melatonin. Severing SCG input into the pineal abolished the rise in melatonin synthesis and c-*fos* transcription suggesting that expression of this proto-

oncogene is functionally linked to indole metabolism in the rat (Carter, 1990). More recently, induction of the c-*fos* gene in the rat pineal was demonstrated to occur primarily through stimulation of $\alpha_1$-adrenoceptors (Carter, 1992).

The density of $\alpha_1$-adrenoceptors in rat pinealocyte membranes is comparable to that for $\beta$-adrenoceptors and the former also maintain a high affinity for norepinephrine. Stimulation of $\alpha_1$ receptors alone, both *in vivo* and *in vitro,* with specific $\alpha$-adrenergic agonists did not increase cAMP production, SNAT activity or melatonin synthesis; however, in the face of concomitant $\beta$-adrenergic stimulation these parameters were markedly increased, thus demonstrating the involvement of both classes of receptors in the nighttime rise in melatonin synthesis (for references, see Sugden, 1989). The mechanism underlying $\alpha$-adrenergic amplification of $\beta$-adrenergic-induced melatonin production appears to involve increased intracellular $Ca^{2+}$ concentration, subsequent activation of protein kinase C (PKC) and hydrolysis of phosphatidylinositol. Sugden (1989) suggested that PKC may mediate the potentiation response by phosphorylating a key component of the $\beta$-adrenergic activation pathway, *e.g.* $G_s$ or adenylyl cyclase; however, this hypothesis has yet to be proven.

The role of $\alpha$- and $\beta$-adrenergic receptors in the control of ovine pineal function is less clear than that determined for rodents. Initial *in vivo* experiments suggested that the regulation of melatonin synthesis in sheep differed markedly from that in the rat. Administration of an $\alpha_1$-adrenoceptor antagonist (prazosin) attenuated the nocturnal rise in pineal and serum concentrations of melatonin without altering SNAT or HIOMT activity, suggesting an important regulatory role for this receptor (Sugden *et al.*, 1985). Further, administration of the $\beta$-adrenergic receptor antagonist, propranolol, failed to significantly reduce melatonin synthesis or secretion, suggesting that $\beta$-adrenoceptors played little or no

role in regulating the nocturnal increase in melatonin synthesis. Arendt and coworkers (1985) reported no change in nocturnal melatonin release in sheep following *in vivo* administration of either $\beta$- or $\alpha$-adrenergic antagonists.

Results of subsequent *in vitro* experiments by Morgan and coworkers (1988) almost completely contradicted the *in vivo* data described above. Static incubation of sheep pineal slices with propranolol ($10^{-5}$ M), but not prazosin ($10^{-5}$), reduced norepinephrine-induced production of melatonin by 60 percent, suggesting that $\beta$-adrenoceptors were of primary importance for melatonin synthesis in this species, similar to the situation observed in rats. Interestingly, treatment of the pineal slices with phorbol 12-myristate 13-acetate (PMA; $10^{-5}$ M) provoked a threefold increase in melatonin production over basal levels, suggesting that synthesis of this indole might also be induced by a receptor that is linked to phosphatidylinositol metabolism, as is the $\alpha_1$-adrenoceptor. Similar results were obtained using a dispersed cell, suspension culture preparation of ovine pinealocytes (Howell and Morgan, 1991).

In several recent *in vitro* experiments, the role of $\beta$- and $\alpha$-adrenergic receptors in the regulation of melatonin synthesis in sheep was further explored. Morgan and coworkers (1989a) reported that although regulation of melatonin synthesis appeared to be primarily mediated through the $\beta$-adrenoceptor, $\alpha$-adrenoceptors also played an important role in the process. Administration of a selective $\alpha_1$-agonist, phenylephrine (PE), in the face of tonic stimulation of $\beta$-adrenoceptors by endogenous catecholamines, increased melatonin release from pineal tissue punches with little or no concomitant increase in cAMP suggesting $\alpha_1$-adrenoceptors were involved in melatonin biosynthesis and that the interaction between the two adrenergic receptors occurred at a site distal to adenylyl cyclase. This mechanism is

distinctly different from that described for the rat, in which the activation of $\alpha_1$-receptors resulted in an amplification of the $\beta$-receptor response, observed as an increase in cAMP above that due to $\beta$-receptor stimulation alone.

More recently, Van Camp and colleagues (1991) reported that two intracellular mechanisms may control melatonin release in ovine pinealocytes *in vitro*. Stimulation of pinealocytes with a high concentration of $\beta$-adrenergic agonist (isoproterenol or norepinephrine, 1 $\mu$M) increased melatonin release with a comparable increase in SNAT activity. However, administration of a lower dose of agonist (isoproterenol, 1 nM) provoked half-maximal stimulation of melatonin release in the absence of a comparable increase in SNAT activity suggesting that a low level of $\beta$-adrenergic stimulation increases melatonin release through an SNAT-independent mechanism. In addition, no evidence of $\alpha_1$ adrenergic regulation of SNAT activity or melatonin release was observed; however, administration of a calcium ionophore, A23187, to increase $Ca^{++}$ influx into pinealocytes, stimulated melatonin secretion with no detectable increase in SNAT activity. These data were interpreted to suggest that, in ovine pinealocytes, a strong $\beta$-adrenergic stimulus provokes melatonin synthesis and release through a cAMP-dependent mechanism that is associated with an increase in SNAT activity, whereas, weak $\beta$-adrenergic stimulation invokes melatonin synthesis and secretion through a calcium-dependent mechanism that is independent of SNAT activity. The biological relevance for having two different mechanisms of $\beta$-adrenergic control over melatonin synthesis and secretion is unclear. The failure of $\alpha$-adrenergic stimulation to alter melatonin synthesis in the study by Van Camp and coworkers (1991) is in disagreement with the previously reported results of Morgan and coworkers (1988, 1989a) and was suggested to result from differences in treatment

protocols. In the earlier experiments the $\alpha_1$-antagonist prazosin inhibited melatonin release at 30 min (Morgan *et al.*, 1989a), but not 4 hr (Morgan *et al.*, 1988) after treatment, and in the present experiment pinealocytes were exposed to antagonist for 6 hr prior to melatonin determination. Therefore, it was suggested that $\alpha$-adrenergic receptors are only transiently involved in the regulation of pineal melatonin synthesis in sheep (Van Camp *et al.*, 1991).

Clearly, from the experiments described above, a strong case has been made for $\beta$-adrenergic regulation of melatonin synthesis and SNAT activity in ovine pinealocytes. Further, the regulatory mechanism appears to be similar to that observed in the rat, except that the amplitude of the increase in SNAT activity is enhanced 50-fold in the rat as compared to three to fourfold in sheep. The apparent role of the $\alpha$-adrenergic receptor in the control of ovine pineal function is not well understood, but may involve a transient, SNAT-independent increase in melatonin synthesis shortly after the onset of darkness, perhaps to ensure a rapid increase in systemic melatonin concentration.

Although research investigating the mechanisms regulating melatonin synthesis in cows has lagged behind that of rodents and sheep, over the past 5 years the bovine pineal has received an increasing amount of attention because it is readily available and large in size, thus making it a good candidate for cell culture. Autoradioghraphic studies of bovine pineal tissue indicate that there are at least four types of catecholamine receptors present ($D_1$ and $D_2$ dopamine, and $\beta_1$- and $\alpha_1$-adrenergic), and that receptor density and distribution varies markedly among the receptor types (Simonneaux *et al.*, 1991). The maximal number of binding sites ($B_{max}$, fmol/mg protein) was greatest for dopamine $D_1$ receptors (499), followed by that for $\alpha_1$ (85), $D_2$ (22.4) and $\beta_1$(10.8) receptors, respectively. Because the density of $\beta$-adrenergic receptors was very low and dopaminergic $D_1$ receptors very high,

it was suggested that regulation of melatonin synthesis in the bovine pineal might involve dopaminergic stimulation and thus differ markedly from that thought to occur in the rat and sheep. Interestingly, in spite of reduced $\beta$-adrenergic receptor numbers, $\beta$-adrenergic stimulation of bovine pinealocytes *in vitro* increased melatonin synthesis concomitant with a two to fivefold increase in SNAT activity (Chan and Ebadi, 1980), similar to that reported for sheep (Namboodiri *et al.*, 1985). This latter observation suggests that there may be some commonality in the regulation of SNAT activity among cows and sheep.

Subsequent investigation of the regulation of melatonin synthesis in bovine pinealocytes in monolayer culture demonstrated that $\beta$-adrenergic stimulation (norepinephrine or isoproterenol) significantly increased cAMP accumulation and melatonin secretion (Rüppel and Olcese, 1991) as has been described for other species. Additionally, administration of a range of isoproterenol ($\beta$ agonist) concentrations in combination with phenylephrine ($\alpha_1$ agonist) failed to reveal a synergistic effect of these two agonists on cAMP production or melatonin synthesis as occurs in the rat. However, it should be remembered that research using ovine pinealocytes has indicated that regulation of melatonin synthesis through $\alpha_1$-adrenoceptors may be transient, occurring shortly after initial exposure and disappearing by 4 hr (Morgan *et al.*, 1988, 1989a). In the present experiment (Rüppel and Olcese, 1991), pinealocytes were exposed to the agonists for 8 hr, and as a result a subtle transient effect might have been masked.

Although it appears that SNAT activity in bovine pineal tissue is regulated, at least in part, through $\beta$-adrenergic pathways, the presence of a high concentration of dopamine $D_1$ and $D_2$ receptors (Simonneaux *et al.*, 1991) has provoked research into a possible role for this catecholamine in the regulation of melatonin synthesis and more specifically SNAT

activity. Almost 25 years ago, Axelrod and coworkers (1969) reported that dopamine stimulated the synthesis of melatonin in rat pineal glands *in vitro*. More recently this catecholamine has been shown to both inhibit (.1 $\mu$M) and stimulate (10 $\mu$M) SNAT activity in intact rat pineal glands in short term culture (Govitrapong *et al.*, 1989). Govitrapong and coworkers (1989) determined that dopamine was present in bovine pineal tissue in concentrations greater than that of norepinephrine (4 vs 2 $\mu$g/gram of tissue). Unfortunately, to date, no studies have been conducted to evaluate the effects of dopamine on SNAT activity in this species and, therefore, the role of this catecholamine in the regulation of melatonin synthesis awaits elucidation.

Indirect evidence, such as the presence of high affinity muscarinic adrenergic receptors (Phansuwan-Pujito *et al.*, 1989) and a specific choline acetyltransferase (Phansuwan-Pujito *et al.*, 1990), supportive of cholinergic innervation of the bovine pineal led some researchers to examine the possibility of muscarinic regulation of SNAT activity. Treatment of pineal explants with the cholinergic agonists (10 $\mu$M) methacholine, carbachol or oxotremorine inhibited basal SNAT activity and the inhibitory effects could be overcome by the addition of muscarinic cholinergic receptor antagonists (20 $\mu$M), atropine or quinuclidinyl benzilate, suggesting that SNAT activity in the bovine pineal may be negatively regulated by a muscarinic cholinergic receptor-dependent mechanism (Pujito *et al.*, 1991).

Hydroxyindole-O-methyltransferase

Although the SNAT-mediated conversion of serotonin to N-acetylserotonin is believed to be the rate limiting step in the synthesis of melatonin, another potential site of regulation is the subsequent conversion of N-acetylserotonin to melatonin by HIOMT. However, as described previously, because this methyltransferase does not exhibit a marked

increase in activity during the scotophase, it is not thought to play a significant regulatory role in the production of melatonin (Reiter, 1991b). Interestingly, in the rat, HIOMT activity is regulated by adrenergic stimulation, however, the negative effects of impaired adrenergic input (exposure to constant light or removal of SCG) occur gradually over days or weeks and daily adrenergic stimulation of the pineal by increased nocturnal secretion of norepinephrine is sufficient to maintain high levels of the enzyme in this species (Sugden, 1989).

Because nocturnal SNAT activity is not dramatically increased in the sheep pineal, it was proposed that HIOMT might participate in the regulation of melatonin synthesis (Namboodiri *et al.*, 1991). Further, *in vivo* administration of prazosin to sheep (3 mg/animal) reduced pineal content of melatonin with no significant decrease in the content of N-acetyltransferase or reduction in SNAT activity (Sugden *et al.*, 1985), suggesting that the effect was mediated through HIOMT; however, no decrease in HIOMT activity was observed. Namboodiri and coworkers (1991) pointed out that these results do not necessarily exclude a regulatory role for HIOMT, because if the enzyme were regulated by allosteric or covalent modifications that do not persist in broken cell preparations no increase in HIOMT activity would be observed. Covalent modification of HIOMT has been reported (Deguchi and Barchas, 1971; Sugden and Klein, 1987).

Research concerning the activity of bovine HIOMT has focused on experiments that assessed the ability of putative regulatory compounds to modify the activity of the purified enzyme. Nanomolar concentrations of estrone, estradiol and progesterone, in order of increasing magnitude, competitively inhibited N-acetylserotonin methylation by bovine HIOMT *in vitro* (Morton and Forbes, 1989), suggesting that ovarian hormones might play

a role in regulating the synthesis of melatonin and other methoxyindoles (*e.g.* 5-methoxyindoleacetic acid, 5-methoxytryptophol). Although no research has been conducted to examine this possibility in the cow *in vivo*, variations in pineal content of melatonin, attributed to HIOMT activity, have been observed during the estrous cycle of the rat (Johnson *et al.*, 1982) and estradiol was found to stimulate HIOMT activity in the guinea pig pineal (Cardinali *et al.*, 1986).

**Target Sites and Mechanism of Melatonin Action**

Once it was clear that photoperiod mediated its effects on mammalian reproduction through the secretion of melatonin, the search for potential target sites and possible modes of action was hastily begun. Initial attempts to determine the site of action focused on the use of receptor binding assays to probe homogenates prepared from suspected target tissues, primarily the brain and reproductive organs, for specific high affinity melatonin binding sites. Early research in this area relied on the use of tritiated melatonin (specific activity 45-80 Ci/mmol) and produced results that were difficult to replicate. The recent development of a high specific activity (2000 Ci/mmol), radioiodinated form of melatonin (2-[$^{125}$I]-melatonin) revitalized interest in the identification and characterization of putative melatonin receptors and results from studies using this radioligand appear to be more reproducible (for review, see Morgan and Williams, 1989; Krause and Dubocovich, 1991; Stankov *et al.*, 1991a,b; Weaver *et al.*, 1991). Although numerous reviews and papers exist that support the existence of a high affinity membrane bound melatonin receptor, it is important to note that some researchers are not convinced that the reported melatonin binding sites represent

actual high affinity receptors because the signal transduction pathway and subsequent cellular response have not been identified and characterized (Kennaway and Hugel, 1992a).

***Central Nervous System***

The majority of work concerning the characterization of melatonin binding sites in the central nervous system (CNS) has involved rodents (for review, see Stankov *et al.*, 1991a; Kennaway and Hugel, 1992b); however, there are several reports describing the distribution of these binding sites in the brains of domestic livestock species, including sheep (Bittman and Weaver, 1990; de Reviers *et al.*, 1991; Stankov *et al.*, 1991b; Helliwell and Williams, 1992), horses (Stankov *et al.*, 1991b), goats (Deveson *et al.*, 1992) and cows (Cardinali *et al.*, 1979). Using autoradiography, a high concentration of melatonin binding sites has been observed in SCN (site of an endogenous circadian pacemaker) and pars tuberalis (PT) of rodents. Other areas with demonstrable binding, albeit less than that in SCN and PT, include the area postrema, several cortical structures, hippocampus and paraventricular thalamic nuclei (for references, see Stankov *et al.*, 1991a).

Mostly similar results have been reported for sheep, suggesting some commonality in the mode of melatonin action among species. Bittman and Weaver (1990) reported that in ewe brains, the highest concentration of melatonin binding sites, as determined by *in vitro* autoradiography, was located in the PT, confirming an earlier report by Morgan and coworkers (1989d). In contrast to rodents, the presence of melatonin binding sites in the sheep SCN is in dispute because some researchers have failed to observe binding sites for the indole in this tissue (Bittman and Weaver, 1990; Weaver *et al.*, 1991; de Reviers *et al.*, 1991) while others have reported the presence of a significant number of melatonin receptors in the SCN of sheep and horses (Stankov *et al.*, 1991b). More recently, melatonin binding

sites were observed in SCN of dairy goats (Deveson *et al.*, 1992). Among the various studies, the apparent discrepancy in the localization of melatonin binding sites in ovine SCN is not readily explainable; however, the distribution of melatonin binding sites in other areas of the brain such as the hypothalamus, preoptic area, hippocampus and cortex, as well as the lack of binding sites in pars distalis and pineal gland, appears to be similar among sheep, horses and goats (Bittman and Weaver, 1990; Stankov *et al.*, 1991b; Deveson *et al.*,1992).

The presence of melatonin binding sites in the anterior hypothalamus and preoptic areas of seasonal breeding domestic livestock is significant because these are associated with the regulation of gonadotropin secretion (Lehman *et al.*, 1986). Helliwell and Williams (1992) reported significant specific labeling of the medial edge of the diagonal band of Broca in the ewe brain, an area known to contain GnRH cell bodies (Caldani *et al.*, 1988). Collectively, the presence of melatonin binding sites in the ovine hypothalamus, preoptic area and diagonal band of Broca suggests that these areas may be sites at which melatonin acts to mediate the effects of photoperiod on reproduction, presumably through the modulation of GnRH secretion. Melatonin binding sites have also been localized to the medial basal hypothalamus (MBH) of the ram (Stankov *et al.*, 1991b) and placement of microimplants of melatonin in the MBH, but not POA or lateral hypothalamus, of OVX estradiol-treated ewes exposed to long days (16L:8D) increased LH secretion in a majority of ewes (7 of 12) similar to that observed for untreated ewes under short days (8L:16D) and ewes receiving a subcutaneous melatonin implant in the ear (Malpaux *et al.*, 1993). These data suggest that, in this species, the MBH may be an important site of melatonin action in the regulation of LH secretion.

Controversy surrounding the presence or absence of melatonin binding sites in the SCN of sheep still needs to be resolved; however; this region may be vital for normal reproductive cycles in ewes because bilateral destruction of the SCN during the breeding season extended the period of cyclicity and ovulation into the non-breeding season in a majority (4 of 6) of ewes (Przekop and Domański, 1980). Ewes subjected to frontal hypothalamic deafferentation (FHD) at the level of the SCN during anestrus failed to display any effect of the procedure on the onset or termination of the first breeding season but 50 percent of the animals began cycling prior to the summer solstice preceding the second breeding season and either cycled continuously or erratically thereafter depending on the precise location of the lesions (Jackson *et al.*, 1986). Further, ewes subjected to FHD during the breeding season either cycled continuously or infrequently. Continuous cycles were observed in ewes with extensive damage to the SCN and adjacent regions whereas ewes with bilateral lesions located between the SCN and the arcuate nucleus primarily displayed reproductive activity similar to that of control sham-operated ewes or that was erratic in nature. Interestingly, FHD did not disrupt the diurnal secretion of melatonin and failed to alter seasonal changes in systemic prolactin concentrations. It was suggested that deafferentation disrupted seasonal reproductive activity in the ewe by interfering with melatonin responsive regions that control LH secretion or the neural pathways that link these putative sites to the GnRH pulse generator. Collectively, data from the lesion experiments tend to support the concept that the ovine SCN is involved in the control of seasonal breeding in the ewe; however, further studies are required to confirm these results because in such experiments, it is difficult to assess the impact of minor tissue damage to neural sites adjacent to target tissue lesions. Zucker and coworkers (1991) have provided an excellent

review on the role of the SCN in the control of annual reproductive and behavioral rhythms of mammals.

In all mammals studied to date, the pars tuberalis of the adenohypophysis contains a high concentration of melatonin binding sites. In sheep, and several other species, the number of melatonin binding sites in the PT exceeds that observed in other regions of the CNS (for references, see Morgan and Williams, 1989; Stankov *et al.*, 1991a,b; Weaver *et al.*, 1991). Because of the size and relative ease of separation from the adenohypophysis, and the high density of melatonin binding sites, the ovine PT has received a great deal of attention in the quest to characterize the functional properties of the putative melatonin receptor. Additional studies have examined the ultrastructure of the pars tuberalis in an effort to identify the melatonin-responsive cell type and to determine the functional role of these cells in mediating the photoperiodic message that controls reproduction in sheep.

At least two cell types, follicular (non-glandular) and secretory (glandular), comprise the mammalian pars tuberalis (Dellman *et al.*, 1974). The ovine PT is primarily comprised of two distinct types of secretory cells, distinguished by the abundance of dense-core granules, but contains few follicular cells (Morgan *et al.*, 1991b). The majority of secretory cells (87%) are characterized by the absence or low abundance of dense-core granules and are arranged in clusters that form a columnar configuration. The remaining secretory cells (10 to 15%) are characterized by an abundance of dense-core granules. Of the two cell types, the predominant agranular cell responds to melatonin stimulation *in vitro* and is presumed to be the endogenous site of melatonin action in this tissue (Morgan *et al.*, 1991b). Interestingly, PT cells of the Djungarian hamster exhibit photoperiod-induced changes in ultrastructure (Wittkowski *et al.*, 1984); however, in the present study no differences in

ultrastructure were observed in ovine PT collected under winter or summer photoperiods. Acceptance of the PT as a site of melatonin action in the control of seasonal breeding will require the characterization of specific melatonin receptors, identification of the mechanism of action and subsequent cellular product(s) generated in response to the indole (for review, see Wittkowski *et al.*, 1992).

Melatonin binding sites in ovine PT were first localized and characterized by Morgan and coworkers (1989d). Autoradiographical localization and binding characteristics of the putative receptor sites in PT homogenates were defined using 2-[$^{125}$I]-melatonin. Radiolabeling was discretely localized to the PT and could be displaced by excess unlabeled melatonin. Binding of radiolabeled melatonin to PT homogenate depended on membrane concentration, time and temperature of incubation. Evaluation of the equilibrium dissociation constant ($K_d$ = 32.5 pM) and maximal number of binding sites ($B_{max}$ = 103$\pm$14 fmol/mg protein) suggested the presence of a high affinity, low capacity receptor for melatonin. Comparable dissociation constants have been reported by others (Sugden and Chong, 1991; Helliwell and Williams, 1992); however, among these studies, $B_{max}$ concentrations (fmol/mg protein) were more variable, with slightly lower (76.3$\pm$2.2; Sugden and Chong; 1991) and greatly higher (217.5$\pm$3.2; Helliwell and Williams, 1992) values reported. One study, reported $K_d$ (17 pM) and $B_{max}$ (20 fmol/mg protein) values that were considerably lower than the previously reported values (Stankov *et al.*, 1991b); however, the apparent discrepancy was not mentioned and possible causes were not addressed.

Differences in binding affinity and the number of binding sites may reflect differences in physiological states, arising from differences in sex, breed, season of the year or other factors, however; Helliwell and Williams (1992) reported no difference in either

affinity or number of melatonin binding sites in ewes during estrus or the luteal phase of the cycle and Sugden and Chong (1991) utilized PT tissue from sheep of mixed sex and breed. Morgan and coworkers (1989d) used PT from anestrus ewes to conduct their binding studies; however, neither sex nor breed was reported for the tissue used in the autoradiography studies appearing in the same publication. Because the influence of sex, breed and season has not been formally investigated and these parameters are not always included in published reports, it is difficult to draw any conclusions about the effects of these factors on the binding characteristics of the putative melatonin receptor. Alternatively, differences between the various research groups might arise from the use of different methods to estimate $K_d$ and $B_{max}$.

Although the pharmacological characteristics of the ovine PT melatonin binding site are suggestive of a high affinity melatonin receptor, the signal transduction pathway and subsequent cellular response associated with the stimulation of this putative receptor have yet to be determined. Early research on the mechanism of melatonin action revealed that melatonin inhibited forskolin-induced, but not basal, cAMP production in ovine PT cells cultured *in vitro* (Morgan *et al.*, 1989c). Further, the ability of 2-[$^{125}$I]-melatonin to bind to membranes prepared from ovine PT was inhibited in a dose-dependent fashion by the addition of guanine nucleotides and related analogues (Morgan *et al.*, 1989b) suggesting that the putative receptor was linked to an inhibitory G-protein. Subsequent experiments revealed that melatonin-induced inhibition of forskolin-stimulated adenylyl cyclase activity (1 $\mu$M forskolin + 1 $\mu$M melatonin) was attenuated, but not completely abolished, in a dose-dependent manner upon incubation with various concentrations (.0005 to .5 $\mu$g/ml) of pertussis toxin (Morgan *et al.*, 1990) suggesting that the putative receptor interacted with a

pertussis toxin-sensitive G-protein. Analysis of ligand binding assays revealed that preincubation of PT membrane homogenates with pertussis toxin (20 $\mu$g/ml) or GTP (1 mM) decreased 2-[$^{125}$I]-melatonin binding by 20 and 40 percent, respectively. Addition of both compounds resulted in an additive reduction (60%) in melatonin binding leading these investigators to speculate that the putative melatonin receptor was functionally coupled to adenylyl cyclase through two distinct G-proteins, one sensitive and the other insensitive to pertussis toxin. An alternative explanation proposed that the receptor existed as two subtypes, each separately linked to distinct G-proteins that may be distinguished by their susceptibility to ADP ribosylation by pertussis toxin.

Because a receptor-mediated stimulus of adenylyl cyclase activity in melatonin responsive PT cells has not yet been identified, research investigating the signalling pathway(s) involved in the cellular response to the indole has relied on the use of pharmacological agents that exert their actions at sites distal to the receptor. Several experiments that further explored the interaction of forskolin and melatonin (Morgan *et al.*, 1991c) and the effect of melatonin on cAMP-dependent protein kinase (PKA; Hazlerigg *et al.*, 1991) in ovine PT cells have been reported. Addition of forskolin, at concentrations greater than 1 $\mu$M, to ovine PT cells provoked a dose-dependent increase in cAMP production that was significantly greater than basal levels and as reported previously (Morgan *et al.*, 1989c) melatonin (1 $\mu$M) inhibited the forskolin response at all concentrations tested (Morgan *et al* 1991c). To assess any potential interactions between different signal transduction pathways, PT cells were incubated with the phorbol ester, phorbol 12-myristate, 13-acetate (PMA), and cAMP production was determined (Morgan *et al.*, 1991c). Administered alone, PMA (10 $\mu$M) induced a small, but significant, increase

in the cyclic nucleotide; however, concomitant exposure of PT cells to forskolin (1 $\mu$M) and PMA (10 $\mu$M) resulted in a strong potentiation of the forskolin response by PMA. Melatonin (1 $\mu$M) attenuated both the non-potentiated (90%) and potentiated (80%) responses. These data were interpreted to suggest that both the cAMP and phospholipase C signal transduction pathways are involved in the inhibitory action of melatonin on ovine PT cells, and that the indole may interrupt cellular processes that arise from the activation of either pathway. In a later study melatonin had no effect on basal or $AlF_4^-$-stimulated phosphoinositide turnover or intracellular $Ca^{2+}$ concentrations (Morgan *et al.*, 1991a) suggesting that the phospholipase C pathway is not directly coupled to the putative melatonin receptor.

Because melatonin inhibits the forskolin-induced production of cAMP in ovine pars tuberalis cells, Hazlerigg and coworkers (1991) explored the possibility that the negative effects of the indole are mediated through an alteration in PKA activity. Forskolin (1 to 100 $\mu$M) activated PKA in a dose-dependent manner. Activation of PKA by forskolin, at concentrations up to 10 $\mu$M, was prevented by the concomitant addition of melatonin (1 $\mu$M) and at a higher concentration of forskolin (100 $\mu$M) the indole markedly reduced the level of activation. Addition of melatonin alone had no effect on PKA activation. Time-course studies of PKA activation by forskolin and inactivation by melatonin revealed that PKA activation was 80 percent of maximum within 2 min and reached a plateau by 10 min after stimulation with the diterpine. Addition of melatonin after preincubation of cells with forskolin for 15 min resulted in a progressive inactivation of PKA which was maximal 10 min after treatment with the indole and showed no further decline 20 min later. Ovine PT cells appear to contain both isoforms of PKA (PKA I and PKA II) because specific

photoaffinity labeling of PKA in unstimulated cell extracts, followed by SDS-PAGE, yielded 3 bands that were consistent in molecular weight with that of the regulatory subunits of PKA I and the phosphorylated and unphosphorylated forms of PKA II. From these data it was concluded that melatonin not only prevents the forskolin-induced activation of PKA in ovine pars tuberalis cells, but can reverse activation of the kinase as well. In addition, because the function of PKA is to phosphorylate specific cellular proteins, it was suggested that melatonin may mediate its effects on this tissue through the dephosphorylation of specific regulatory proteins.

In addition to the lack of information concerning the putative endogenous agonist that stimulates adenylyl cyclase in ovine pars tuberalis cells, research investigating the cellular consequences of melatonin action in this tissue has also been hampered by the lack of information regarding the synthesis and secretion of products by PT cells following exposure to the indole. Morgan and coworkers (1992) investigated protein synthesis and secretion in primary cultures of ovine pars tuberalis cells by incubating cells with [$^{35}$S]methionine in the presence or absence of forskolin (10 $\mu$M), melatonin (1 $\mu$M) or forskolin + melatonin and labeled cellular and secreted proteins were separated using gel electrophoresis (SDS-PAGE). Forskolin alone stimulated the accumulation of 9 proteins (range = 14 to 72 kDa) in the medium with no corresponding changes in cellular proteins. Melatonin inhibited the forskolin-induced synthesis and accumulation of the secreted proteins with the exception of the 23 kDa protein. Pulse-chase studies revealed that a majority of secreted proteins (72, 62, 44, 39, and 23 kDa) appeared in the cell within 5 min of forskolin treatment but did not appear in the medium for an additional 25 min. It was suggested that the time-lag was due to intracellular processing and packaging events that are associated with secretory proteins.

Again, with the exception of the 23 kDa protein, melatonin inhibited forskolin-mediated synthesis and secretion of proteins from PT cells. Regulation of the synthesis and secretion of the melatonin-sensitive secretory proteins appears to occur at the level of translation because administration of actinomycin D (1 $\mu$g/ml; transcription inhibitor) failed to reduce basal or forskolin-induced protein secretion. In contrast, actinomycin D markedly attenuated the synthesis of the melatonin-insensitive 23 kDa protein. Further, similar experiments conducted with primary cell cultures of pars distalis (PD) cells revealed that many of the forskolin-induced secretory proteins of the PT are similarly induced in the PD; however, the inhibitory effects of melatonin were specific to PT cells. The only secretory protein to be successfully identified was the 23 kDa protein which was identified as prolactin. From these data it was suggested the forskolin-induced, melatonin-insensitive synthesis and secretion of prolactin represented the action of the diterpine on a population of non-melatonin responsive cells in the ovine PT. Further, because melatonin did appear to regulate the synthesis and secretion of several PT proteins that were also common to PD cells, it was speculated that these proteins might function in the process of secretory protein exportation from various cells including those of the PT and PD. Because no PT-specific protein was detected it was proposed that melatonin may induce the synthesis of a non-protein secretory product or that the induced proteins lack methionine and therefore were not detected by the methods used in these experiments.

In a recent publication, long term exposure (16 hr) of PT cells to physiological concentrations of melatonin (100 pM) was reported to sensitize adenylyl cyclase, resulting in an increase in both basal and forskolin-stimulated production of cAMP that did not depend on *de novo* protein synthesis (Hazlerigg *et al.*, 1993). In addition, extended melatonin

treatment (24 hr) resulted in the down-regulation of melatonin binding sites; however, this reduction was not associated with a decrease in the ability of melatonin to inhibit forskolin-induced cAMP production, suggesting that PT cells contain an excess of the binding sites. Further, based on these *in vitro* results, the authors have suggested that similar changes may occur in sheep, *in vivo*, resulting in the sensitization of the PT during winter photoperiods (reduced photophase) in response to the extended duration of the nocturnal rise in melatonin and the subsequent desensitization during summer photoperiods (extended photophase) when exposure to the nocturnal increase in melatonin is reduced ($<8$ hr). The finding that melatonin binding sites in the PT are down-regulated by extended exposure to the indole *in vitro* is interesting because it suggests that photoperiod may regulate the sensitivity of this tissue to melatonin. In support of this hypothesis, extended exposure to light, increased the apparent number of melatonin binding sites in the PT of rams (Pelletier *et al.*, 1990).

Clearly, there are still many questions concerning the nature and function of the putative melatonin receptor in the pars tuberalis, and other areas of the CNS, that remain to be answered if we are to completely understand the underlying mechanism whereby melatonin mediates the photoperiodic regulation of reproduction in mammals. Although functional melatonin binding sites have been expressed in Xenopus oocytes following injection of poly($A^+$) RNA extracted from ovine PT (Fraser *et al.*, 1991), the putative receptor has yet to be cloned and expressed in mammalian cell lines. In addition, research investigating the mechanism of action of melatonin, specifically in ovine PT, has been hampered by the inability to identify the endogenous receptor agonist that stimulates the cAMP signal transduction pathway that is negatively regulated by melatonin.

Based on the collective results of research conducted with ovine PT cells, Morgan and coworkers (1991a) proposed that the inhibitory effects of melatonin on cAMP production are mediated through a complex multi-receptor pathway that involves two yet unknown stimulatory receptors and the putative melatonin receptor. One stimulatory receptor is believed to be coupled to the adenylyl cyclase-cAMP pathway that melatonin actively inhibits. The other receptor is thought to be coupled to a melatonin-sensitive phospholipase C-phosphoinositide pathway that stimulates cAMP synthesis through the activation of PKC. The putative melatonin receptor is also presumed to be coupled to adenylyl cyclase but through pertussis toxin sensitive and insensitive G proteins.

Regrettably, only one early study exists that characterized the presence of melatonin binding sites in bovine brain (Cardinali *et al.*, 1979). In this study, [$^3$H]-melatonin was reported to bind specifically and with high affinity ($K_d = 1.2 \pm .4 \times 10^{-8}$) to membrane preparations from the medial basal hypothalamus ($B_{max} = 6.5 \pm .8$ fmol/mg protein), occipital cortex ($B_{max} = 4.8 \pm .6$ fmol/mg protein) and cerebral cortex ($B_{max} = 2.2 \pm 1.1$ fmol/mg protein). Although this study demonstrated high affinity binding sites in several bovine brain regions, in light of the relatively high $K_d$ value that was obtained and the current availability of the high specific activity melatonin agonist, 2-[$^{125}$I]-melatonin, new studies should be conducted to fully characterize and localize melatonin binding sites in bovine brain and pars tuberalis. Results from autoradiographical localization and ligand binding experiments would likely reveal additional binding sites that could not be detected using the tritiated ligand and may allow a more accurate determination of $K_d$ values. Subsequent findings could then be compared with similar data obtained for seasonally breeding farm species.

***Ovary and Testis***

Although the effects of melatonin on reproduction in mammals are believed to be mediated through the alteration of hypothalamic GnRH secretion, there is evidence to support a peripheral action of melatonin on gonadal tissues of some mammalian species. In hypophysectomized, human chorionic gonadotropin (hCG; .1 IU every 2 days for 22 days) - treated male rats, melatonin (750 $\mu$g, s.c. daily for 22 days) significantly reduced testis and ventral prostate weights (Debeljuk *et al.*, 1971) suggesting a direct action of the indole on these tissues. Subsequently it was reported that melatonin inhibited androgen production from rat testis *in vitro* (Ellis, 1972).

Similarly, a direct effect of melatonin on human ovarian tissue *in vitro* was also reported (Macphee *et al.*, 1975). In this study, melatonin (50 nmol) stimulated progesterone synthesis, from $^{14}$C-1-acetate, in CL slices and the response to various concentrations of the indole ($1.5 \times 10^{-5}$ to $1 \times 10^{-8}$ M) was dose-dependent. Melatonin also increased basal progesterone, but not estrogen, production in rat granulosa cells *in vitro* and the responsiveness of the cells to melatonin appeared to be potentiated by gonadotropins (Fiske *et al.*, 1984). Several recent experiments have demonstrated that melatonin increases basal (Webley and Luck, 1986) and hCG-stimulated (Brzezinski *et al.*, 1992) progesterone, but not estradiol, synthesis in human granulosa cells and, similar to that reported for rats, melatonin potentiated the response of granulosa cells to hCG (Brzezinski *et al.*, 1992).

Melatonin has also been shown to increase progesterone synthesis by bovine (Webley and Luck, 1986) and ovine (Baratta and Tamanini, 1992) granulosa cells *in vitro*, suggesting that the indole may have a direct effect on follicular function in these species. Webley and Luck (1986) reported a significant increase in progesterone production in response to the

addition of physiological concentrations of melatonin (100 to 400 pg/ml) to bovine granulosa cells isolated from preovulatory follicles and maintained in culture for 24 hr. In contrast, melatonin ($10^{-5}$ to $10^{-7}$ M) had no effect on progesterone production by bovine luteal cells incubated *in vitro* for 2 hr (Battista and Condon, 1986) suggesting that ovarian sensitivity to the indole might be exclusive to granulosa cells in this species. Baratta and Tamanini (1992) reported that melatonin (.86 to 86 nmol/L) failed to stimulate progesterone production in ovine granulosa cells in short-term culture (1 to 2 hr) or in luteal cells in long- (9 days) or short-term cultures. In the case of long-term incubation of ovine granulosa cells, exposure to melatonin (.86 nmol/L) beginning on day 2 or day 6 of culture, and continuing for 4 days, significantly increased progesterone production on day 2 and days 6 and 7; however, progesterone levels were significantly greater on days 6 and 7 than on day 2. Additionally, in the presence of ovine LH (oLH; 2 ng/ml) exposure of cells to melatonin, beginning on day 2 of culture, significantly potentiated the melatonin-induced stimulation of progesterone on days 3, 4 and 5. As was reported for the rat (Fiske *et al.* 1984) and human (Webley and Luck, 1986; Brzezinski *et al.*, 1992) melatonin failed to alter estradiol production by ovine granulosa cells. Although the physiological significance of melatonin-induced production of progesterone by granulosa cells is not known, Baratta and Tamanini (1992) speculated that, in the ewe, increasing exposure to endogenous melatonin during the fall may stimulate enhanced follicular synthesis of progesterone which may aid in the induction of estrous cycles at the start of the breeding season. In non-seasonal breeding species such as rats, cows and humans the significance is less clear, although collectively these data suggest that melatonin may play a role in modulating ovarian function. Interestingly, melatonin is present in human follicular fluid, at levels that are three times

greater than occurs in serum (Brzezinski *et al.*, 1987). More recently, it was reported that follicular fluid, aspirated from preovulatory follicles of Finnish women living under the natural photoperiodic conditions of extended hours of daylight (spring to fall) and darkness (fall to spring) that are associated with extreme northern latitudes, exhibits both circadian and seasonal variations in melatonin concentration (Rönnberg *et al.*, 1990).

Following the early demonstration of the direct effects of melatonin on rat testis and human ovary, Cohen and coworkers (1978) investigated the possibility that gonadal tissue might exhibit specific receptors for the indole. These researchers reported the presence of cytosolic melatonin binding sites in hamster, rat and human ovary, and rat testis. The $K_d$ for the high affinity binding sites was estimated to be $6.3 \times 10^{-9}$ M. Additional tissues, from rats and hamsters, exhibiting significant cytosolic [$^3$H]-melatonin binding sites included uterus, liver and skin. These data have been viewed with some skepticism because more current research characterizing melatonin binding sites in the brain and pars tuberalis has suggested that the putative melatonin receptor is membrane bound (Stankov *et al.*, 1991a,b). More recently, Helliwell and Williams (1992) reported no specific 2-[ $^{125}$I]-melatonin binding in the ovary, thyroid, adrenals, liver, uterus or skin of sheep. In contrast, specific high affinity 2-[ $^{125}$I]-melatonin binding sites have been identified in membrane preparations from chicken testes and ovaries (Ayre *et al.*, 1992) suggesting that the indole may have a direct gonadal effect in this species.

Because there are few published reports on the distribution of melatonin binding sites in mammalian gonads and high specific activity melatonin agonists are readily available, the search for putative melatonin receptors in various reproductive tissues should be repeated using more modern approaches and extended to represent a greater number of species.

Should a thorough investigation reveal the absence of gonadal melatonin binding sites, alternative modes of melatonin action should be explored. For example, melatonin, a lipophilic compound, may enter target cells by passive diffusion and subsequently participate in non-receptor mediated events or, alternatively, might be metabolized into a specific compound that alters cellular function. As is the case with melatonin target sites in the CNS, the importance of the observed effects of the indole on gonadal function will remain controversial until a specific melatonin receptor-effector system or an alternative intracellular biochemical pathway is definitively identified.

**Melatonin: The Endocrine Code for Daylength**

It is well accepted that melatonin is the endocrine code for daylength and that, in most seasonally breeding mammals, the **duration** of the rise in nocturnal melatonin concentration is the melatonin secretory pattern characteristic that transmits daylength information (Morgan and Williams, 1989; Karsch *et al.*, 1991; Reiter, 1991a,b). The melatonin message may be interpreted with different reproductive consequences depending on the species involved; for example, the Djungarian hamster becomes reproductively active under conditions of lengthening photoperiod (spring), whereas, in the ewe, reproductive cycles are initiated under conditions of decreasing photoperiod (autumn). Because the duration of nocturnal melatonin secretion is proportional to the length of the scotophase (dark period), under the same photoperiod conditions, both species are exposed to elevated nighttime concentrations of melatonin for a similar length of time; however, with opposing effects on reproductive function. Although the precise mechanism whereby melatonin alters reproductive function in different species has not yet been determined, our present

understanding of the role that melatonin plays in the neuroendocrine regulation of annual reproductive cycles has been largely determined from experiments conducted with ewes (for review, see Karsch *et al.*, 1984; Arendt, 1986; Karsch and Moenter, 1990; Karsch *et al.*, 1991; Reiter, 1991b). In addition to controlling the timing of reproductive activity in adult animals, photoperiod, acting *via* melatonin, has also been shown to influence growth and the attainment of puberty in ewe lambs and heifers (for review, see Yellon *et al.*, 1992) and mammary growth in heifers (Petitclerc *et al.*, 1985; Sanchez-Barcelo *et al.*, 1991).

***Synchronization of Annual Reproductive Cycles of the Ewe***

The annual reproductive cycle of the ewe is characterized by the initiation of repeated 16 day estrous cycles in the autumn that persist, in the absence of pregnancy, until late winter at which time ovarian cycles cease and seasonal anestrus begins (Karsch *et al.*, 1984). Early experiments using natural and artificially reversed photoperiods (Thwaites *et al.*, 1965), demonstrated that induction of the breeding season in Southdown ewes was always associated with decreasing, and seasonal anestrous with increasing, daylength; suggesting that photoperiod synchronized the timing of the breeding season in this species. Similar conclusions were drawn from the results of daylength shift experiments (Legan and Karsch, 1980) in which ewes exposed to alternating artificial long- (16L:8D) and short- (8L:16D) day photoperiods every 90 or 120 days ceased cycling after each shift to the long-day photoperiod, whereas shifting to the short-day photoperiod resulted in the return, after a 50 day delay, of normal cycles (Legan and Karsch, 1980).

To determine if the transitions into and out of the breeding season of the ewe were actively driven by the associated changes in photoperiod, ewes were maintained outdoors under natural photoperiod until the winter (Robinson and Karsch, 1984) or summer

(Robinson *et al.*, 1985) solstice at which time they were brought indoors and were exposed to a fixed daylength equivalent to that of the solstice or to a gradual increase or decrease in daylength, respectively, to simulate the naturally occurring photoperiod. These "solstice-hold" experiments demonstrated that "holding" ewes on either the winter or summer solstice photoperiod failed to delay the termination or initiation, respectively, of the breeding season and provided strong evidence that, in this species, the changes in daylength associated with the onset and termination of the breeding season did not actively stimulate or inhibit the reproductive transitions, rather the transitions occurred because the ewes become refractory to the prevailing inhibitory or inductive photoperiod.

Although it was determined that photoperiod did not actively drive the seasonal shifts in reproductive status in ewes, there is now substantial evidence to support the premise that seasonal breeding in this species is driven by an intrinsic circannual rhythm in reproductive function. Several experiments suggested the presence of an endogenous rhythm, because pinealectomy (PNX; Bittman *et al.*, 1983) or blinding (Legan and Karsch, 1983) of ewes, procedures that prevent the perception of changes in photoperiod, failed to abolish the periodic switches between reproductive competence and incompetence. A subsequent experiment in which ewes were subjected to prolonged exposure (5 years) to a fixed inductive (8L:16D) photoperiod clearly demonstrated the presence of an intrinsic rhythm in reproductive function (Karsch *et al.*, 1989). In this study, ewes under the fixed photoperiod displayed cycles of neuroendocrine reproductive function (high systemic levels of LH) that persisted over the 5 year period. Additionally, the occurrence of the cycles became desynchronized among individual animals and out-of-phase with those of ewes maintained under natural photoperiod, as would be expected if the rhythm were inherent. Further, in

the absence of photoperiodic cues, the period of the cycles averaged 329 days as compared with 364 days for the ewes under natural photoperiod.

Having determined that the ewe possessed an intrinsic circannual reproductive rhythm, subsequent experiments focused on determining if photoperiod, through the circadian pattern of melatonin secretion, was responsible for entraining (synchronizing) the rhythm to the seasons of the year. Woodfill and coworkers (1991) reported that PNX-ewes alternately infused (every 6 months for 2.5 years) with melatonin (for 70 days) in a pattern characteristic of short- (16 hr darkness) and long- (8 hr darkness) daylengths exhibited synchronous cycles of reproductive neuroendocrine activity (elevated systemic LH) that averaged 1 year in length ($368 \pm 3$ days). Further, subsequent exposure, at the start of the third year, to one 70 day period of the long-day melatonin infusion pattern was found to be sufficient to maintain the circannual rhythm. From these data it was concluded that the endogenous reproductive rhythm of the ewe is synchronized by photoperiod, acting *via* melatonin, and that only a portion of the photoperiodic cycle need be perceived to entrain the rhythm to a period of 1 year.

The role of specific photoperiods in the timing of seasonal breeding in ewes has also been investigated. Current dogma favors the hypothesis that the onset of the breeding season in the fall is synchronized by exposure to the increasing photoperiod that occurs prior to the summer solstice, whereas, decreasing photoperiod following the summer solstice is requisite to maintain the full duration of the breeding season (Karsch *et al.*, 1991). Wayne and coworkers (1990) pinealectomized estradiol-treated OVX ewes at various times (summer and winter solstices, vernal and autumnal equinoxes) during the annual photoperiodic cycle and determined the effect of the "timed" pinealectomies on the onset, as well as the duration of

the subsequent "endocrine" breeding season, as assessed by elevated systemic levels of LH. In control ewes, LH levels increased in September and decreased to basal levels in January and February. Ewes pinealectomized near the summer solstice (June 12-23; mid-anestrus) experienced the rise in LH associated with the breeding season, in August, earlier than control ewes, and similarly, the decline in LH levels occurred near the end of October, well in advance of the control ewes. Ewes pinealectomized near the autumnal equinox (September 11-October 1; transition to breeding season) demonstrated elevated LH levels beginning in August and September; however, because LH levels had already risen in several ewes prior to PNX, the time of breeding season onset for this group was not compared with that of control ewes. With respect to duration of the LH rise, LH levels fell to baseline 2.5 wk earlier than was observed in control ewes. Pinealectomy of ewes near the winter solstice (December 8-22; late breeding season) failed to effect LH secretion during that breeding season. During the subsequent breeding season, ewes exhibited no difference in the duration of the LH rise as compared to control ewes; however, in one-half of the ewes (3) the onset of the LH rise began very late and, for two of the ewes, was still in progress that April. The remaining ewes (3) failed to exhibit any rise in LH secretion that breeding season. Lastly, ewes pinealectomized near the spring equinox (March 9-April 3; early anestrus) exhibited a marked delay in the rise in LH secretion associated with the onset of the next breeding season compared with control ewes and the fall in LH was also severely delayed with only one ewe exhibiting basal levels of LH by the end of the study in April.

These results confirmed and extended those reported previously by Malpaux and coworkers (1989) and revealed that different portions of the annual photoperiodic cycle

played various roles in the regulation of the timing of the breeding season in the ewe. Wayne and coworkers (1990) concluded that the lengthening days following the winter solstice were necessary to synchronize the onset of the breeding season to the appropriate time of the year, because pinealectomy of ewes at the winter solstice or spring equinox resulted in marked delays in the onset of the LH rise. Further, it was suggested that the decrease in daylength that occurs from the summer solstice to the autumnal equinox is not only important for the timing of the breeding season (suppresses onset until autumnal equinox and maintains normal duration) but is also involved in regulating the intensity of reproductive neuroendocrine induction, because ewes pinealectomized around the summer solstice experienced an advanced onset in the rise of LH that was short in duration, and the amplitude of the rise was significantly suppressed ($\sim$50%) as compared with control ewes. A similar effect of decreasing daylength on the duration of the breeding season has also been reported (O'Callaghan *et al.*, 1991a).

Interestingly, because pinealectomy at the autumnal equinox only weakly affected the duration of the LH rise (2.5 wk advancement) it was suggested that by the time the breeding season begins, the ewe has received the photoperiodic cues that are relevant to the initiation and termination of that breeding season. However, although removal of photoperiodic cues that occur from the autumnal equinox to the winter solstice had little effect on the onset of the impending breeding season, Jackson and coworkers (1988) reported that this portion of the photoperiodic cycle is necessary to end the photorefractoriness to long daylengths, and thus plays a role that, based on the findings of the present study, is critical for proper synchronization of the subsequent breeding season.

Malpaux and coworkers (1989) initially proposed a working model, that was recently updated (Karsch *et al.*, 1991), that describes the role of photoperiod in the temporal regulation of the annual reproductive cycle of the ewe. In this model, an inherent circannual rhythm provides the primary momentum for the occurrence of seasonal reproductive transitions. The endogenous circannual rhythm is synchronized by photoperiod, through the circadian pattern of melatonin secretion. The endocrine signal of the winter-to-spring phase of the year, decreasing duration of melatonin secretion in response to increasing daylength after the winter solstice, synchronizes an internal process that ultimately initiates the onset of the breeding season. The onset of reproductive activity is timed to the appropriate season by the inhibitory long days surrounding the summer solstice, that postpone immediate expression of the breeding season until the early autumn. Subsequently, the endocrine signal for the summer-to-autumn phase of the year, increasing duration of melatonin secretion in response to decreasing daylength following the summer solstice, prolongs the breeding season until the inherent process ultimately signals the transition out of the breeding season and into anestrus. Further, decreasing photoperiod after the autumnal equinox serves to remove the photoinhibition to increasing photoperiod after the winter solstice, thus allowing synchronization of the subsequent breeding season.

***Regulation of the GnRH Pulse Generator in the Ewe***

As described in a previous chapter, the hypothalamic GnRH pulse generator (also referred to as the LH pulse generator) is the neuroendocrine mechanism that gives rise to the pulsatile release of gonadotropins, through pulsatile release of GnRH, that regulate the ovarian cycles of female mammals. It is now well accepted that photoperiod, through the action of melatonin, controls seasonal breeding in the ewe, and presumably other female

mammals, through functional modulation of the GnRH pulse generator. Further, photoperiod has two different types of effects on LH secretion in the ewe, referred to as the **direct photoperiod drive** and **shift in estradiol negative feedback** (for review, see Karsch *et al.*, 1984; Karsch and Moenter, 1990).

Initially, direct photoperiod drive to the GnRH pulse generator was observed as seasonal alterations in the pattern of LH secretion in OVX-ewes maintained under natural photoperiod (Goodman *et al.*, 1982). During the long photoperiod of summer LH secretory profiles were characterized by large, infrequent pulses of LH; however, under the shorter photoperiods of winter, pulses of LH decreased in amplitude and increased in frequency. Clearly, in the absence of gonadal steroids, the anestrous season (summer) is associated with reduced, and the breeding season (autumn) with enhanced, pulsatile secretion of LH. Similar effects were observed using artificial photoperiods (Goodman *et al.*, 1982).

Legan and coworkers (1977) reported that the negative feedback action of estradiol on LH secretion in the ewe varied markedly with the time of the year. Estradiol was a potent inhibitor of LH secretion during anestrus but this effect was reduced during the breeding season. A subsequent experiment (Legan and Karsch, 1980), in which intact and estradiol-treated OVX-ewes were subjected to 90 days of alternating short- and long-day photoperiod provided further support that photoperiod mediates the transitions into and out of the breeding season by modulating the response of the hypothalamic-pituitary axis to estradiol negative feedback. Intact ewes under this treatment regimen underwent two breeding and two anestrous seasons in one year. Coincident with the seasonal transitions of the intact ewes, LH secretion was enhanced at the onset of the breeding season and reduced at the onset of anestrus in estradiol-treated OVX-ewes. However, although these

experiments clearly demonstrated the shift in estradiol negative feedback, the primary site of estradiol action had not yet been determined.

Goodman and coworkers (1982) provided strong evidence to support the concept that the seasonal effect of photoperiod on hypothalamic-pituitary sensitivity to the inhibitory effects of estradiol occurred at the level of the GnRH pulse generator in the hypothalamus rather than at the pituitary. Results of this experiment clearly demonstrated that administration of estradiol during anestrus abolished pulsatile LH secretion without affecting pituitary sensitivity to exogenous GnRH (2 ng/kg body weight), whereas, similar treatment during the breeding season resulted in the persistence of high frequency pulses of LH. Similar results have been reported in ewes subjected to artificial photoperiods (Goodman *et al.*, 1982) and in ewes receiving melatonin infusions that mimicked the pattern of melatonin secretion characteristic of short or long photoperiods (Bittman *et al.*, 1985). Further, results of subsequent experiments specifically designed to evaluate the site of melatonin action in the regulation of LH secretion in the ewe (Robinson *et al.*, 1986), were consistent with the hypothesis that melatonin does not exert its effects on the pituitary but rather acts on the neural elements of the hypothalamus to modulate the pulsatile release of LH.

More convincing evidence to support the hypothesis that the transition from the breeding season to anestrus in ewes involves a change in the secretion of GnRH was recently reported (Barrell *et al.*, 1992). In this study, the pattern of GnRH secretion into pituitary portal blood was determined during the breeding (luteal and synchronized follicular phases) and anestrous (absence of exogenous steroids or following withdrawal of exogenous progesterone) seasons of ovary-intact ewes. The frequency of GnRH release in cyclic ewes during the luteal phase was 1.4 pulses/6 hr and increased to 7.8 pulses/6 hr during the

follicular phase, following progesterone withdrawal (to synchronize follicular phases). In contrast, the frequency of GnRH release in untreated or progesterone-treated anestrous ewes averaged less than 1 pulse/6 hr; however the amplitude of GnRH pulses was greater than that observed in both groups of cyclic ewes. These data provide stronger evidence that the seasonal effect of photoperiod on the neuroendocrine axis of ewes is mediated through an alteration in the frequency of GnRH and, subsequently, LH secretion.

Karsch and coworkers (1984) proposed a model to explain the photoperiodic control of estrous cyclicity in the ewe. The model is based on the hypothesis that transitions between the breeding season and anestrus are determined by the interaction between seasonal changes in photoperiodic drive and intensity of estradiol negative feedback at the GnRH pulse generator. Specifically, during inductive photoperiods (short days) of the breeding season, the pulse generator is under high photoperiodic drive which allows the frequent release of GnRH pulses from the hypothalamus. Further, at this time, the ability of estradiol to suppress GnRH secretion from the hypothalamus is reduced, thus allowing LH pulse frequency to increase. The increase in LH secretion stimulates increased release of follicular estradiol which further enhances LH release until the frequency of pulses is sufficient to provoke ovulation, in the absence of the inhibitory effects of progesterone, and the induction of regular estrous cycles is initiated. In contrast, under inhibitory photoperiods of the anestrous period (long days) there is a low photoperiodic drive to the GnRH pulse generator. Additionally, the hypothalamus becomes highly sensitive to the inhibitory action of estradiol, resulting in a reduction in the pulsatile release of GnRH and thus LH. As a consequence of reduced LH pulse frequency, the gonadotropin is unable to induce the rise

in systemic estradiol that is necessary to induce the preovulatory surge of LH and estrous cycles are inhibited.

Although it is well accepted that seasonal changes in the sensitivity of the hypothalamic GnRH pulse generator to estradiol negative feedback determine the reproductive transitions into and out of the breeding season of the ewe (Karsch *et al.*, 1984), the mechanism underlying this process is not well understood. Several researchers have investigated the possibility that the seasonal alteration in the sensitivity to estradiol may result from changes in the concentrations of receptors for estrogen in neural and(or) anterior pituitary tissues of the ewe (Clarke *et al.*, 1981; Glass *et al.*, 1984); however, the results were equivocal. More recently, Bittman and Blaustein (1990), evaluated the effects of daylength on the concentration of occupied nuclear estrogen and cytosolic progestin receptors in neural (preoptic area, POA; medial basal hypothalamus, MBH; lateral preoptic area, REST-POA; amygdala, AMYG) and anterior pituitary (PIT) tissues of ewes. In this study ewes were subjected to alternating periods (90 days) of long (16L:8D) or short (16D:8L) photoperiods for 290 days to induce breeding or anestrous states. Prior to determination of receptor levels, ewes were ovariectomized and given steroid treatments to mimic a luteal phase followed by either follicular phase levels of estradiol or total withdrawal of exogenous steroids to assure that the recent steroid histories of breeding season or anestrous ewes were comparable. Daylength had no significant effect on the concentration of nuclear estrogen or cytosolic progestin receptors in any of the neuroendocrine tissues examined. Treatment with estradiol did induce a significant increase in the concentration of progestin receptors in POA, MBH and PIT tissues independent of photoperiod. From these data, it was concluded that although photoperiod failed to alter neural or pituitary concentrations of either

steroid receptor, as well as, the induction of the progestin receptor by estradiol, the possibility that such events take place cannot be completely ruled out at this time. It was also suggested that the use of a more sensitive method of receptor determination, involving specific antibodies to progestin and estrogen receptors, may provide further insight toward resolution of this biological question.

Because there is increasing evidence that opioids are involved in the control of LH secretion during the estrous cycles of many mammals and that the negative feedback effects of gonadal steroids may be mediated *via* an opioidergic mechanism (Kalra, 1986), the potential role for opioids in the regulation of LH secretion during the anestrous period of ewes was investigated. Several experiments have demonstrated that administration of an opioid antagonist, naloxone or WIN 44441-3, during the anestrous period failed to enhance LH secretion in untreated intact ewes (Brooks *et al.*, 1986b), estradiol-treated OVX ewes (Brooks *et al.*, 1986a) and OVX ewes treated with progesterone and estradiol (Yang *et al.*, 1988) suggesting that the strong negative feedback effects of basal concentrations of estradiol on LH secretion during the anestrous period are mediated through a non-opioid mechanism. Further, Yang and coworkers (1989b) reported that although administration of melatonin implants to anestrous ewes advanced the onset of the breeding season, the effects of melatonin did not appear to be mediated *via* an opioidergic pathway. Collectively, these data suggest that, in ewes, opioids are most likely not involved in the photoperiod-induced regulation of LH secretion during the non-breeding season.

Catecholamines have also been postulated to play a role in the regulation of GnRH secretion in domestic livestock (for review, see Daily *et al.*, 1987), and therefore it was of interest to determine if biogenic amines were involved in mediating the effects of

photoperiod on LH secretion in the ewe. Goodman and Meyer (1983) reported that administration of pentobarbital (anesthetic) to anestrous ewes increased LH pulse frequency, suggesting that an inhibitory neural mechanism may regulate the pulsatile release of GnRH and thus tonic LH secretion during the non-breeding season. This hypothesis was further tested in a subsequent experiment in which ovary-intact ewes were treated, during the breeding and non-breeding seasons, with antagonists specific for various neurotransmitter receptors (Meyer and Goodman, 1985). Administration of pimozide (.08 mg/kg body weight, i.v.; dopaminergic antagonist) or phenoxybenzamine (.8 mg/kg body weight, i.v.; $\alpha$-adrenergic antagonist) significantly increased LH pulse frequency in anestrous, but not luteal phase, ewes suggesting that catecholamines are involved in the mechanisms that suppress LH secretion during the non-breeding season but not the luteal phase of the estrous cycle. In addition, both antagonists failed to increase LH pulse frequency in similarly treated OVX ewes during the anestrous season, suggesting that catecholaminergic pathways may not be involved in the estradiol-independent (direct photoperiodic drive) suppression of the LH pulse generator. In contrast, enhanced pulsatile LH secretion in response to pimozide, but not phenoxybenzamine, returned when OVX-ewes received estradiol implants 2 days prior to administration of the antagonists. These data suggest that estradiol may act *via* an inhibitory dopaminergic pathway to reduce LH pulse frequency during the non-breeding season and that the adrenergic suppression of pulsatile LH release observed in ovary-intact anestrous ewes may occur independent of the actions of estradiol.

More recently, Scott and coworkers (1992) investigated the role of two catecholamines, noradrenaline (NA; norepinephrine) and adrenaline (A; epinephrine), in the regulation of LH secretion in OVX and estradiol-treated OVX Corriedale ewes during the

breeding and non-breeding seasons. In this study, NA or A (10 $\mu$g) was microinjected (1 $\mu$l) into the septo-preoptic area (S-POA) of the brain, because, in the ewe, this region contains the majority of GnRH cell bodies (Lehman *et al.*, 1986). No difference was observed between the effects of NA or A on LH secretion during either the breeding or non-breeding season. During both the anestrous and breeding seasons, administration of NA/A failed to alter LH secretion in untreated OVX ewes, suggesting that the GnRH pulse generator, in this case, is subserved by endogenous NA/A input, presumably permissive in function, and therefore is unresponsive to further input. Administration of estradiol to OVX ewes during the anestrous season, abolished or markedly reduced pulsatile LH secretion; however, injection of NA/A rapidly reinstated or increased pulsatile LH secretion such that the interval between pulses was similar to that observed in OVX ewes. These data were interpreted to suggest that the strong negative feedback of estradiol during anestrus involves reduced NA input into the S-POA, and that the effect can be overcome by administration of exogenous NA/A. Further, during the breeding season, the effect of estradiol on LH secretion was dose-dependent. Treatment with estradiol implants of .5 cm length, failed to alter basal LH concentrations, however, treatment with 1 cm implants significantly reduced both the amplitude and frequency of LH pulses. In the presence of a low dose of estradiol (.5 cm implant), NA/A significantly decreased the interval between LH pulses (increased pulse frequency) but with a higher dose of estradiol (1 cm implant) the interpulse interval increased (reduced pulse frequency). The basis for and significance of the differential effects of NA/A in response to varying doses of estradiol was not determined. However, the authors suggested that the differential response might reflect an important physiological

switch; under conditions of low systemic estradiol concentration, NA input is stimulatory, whereas, at higher levels it is inhibitory.

Collectively, these data provide evidence that noradrenergic/adrenergic pathways, at the level of the septo-POA, play a role in the regulation of GnRH secretion in the ewe. In addition, it was suggested that NA/A systems may be involved in the seasonal shift in the negative feedback of estradiol on pulsatile GnRH secretion. The strong negative feedback of estradiol on GnRH secretion during the non-breeding season may occur in response to the withdrawal of permissive NA/A inputs. The role of NA/A systems in the regulation of GnRH secretion during the breeding season is less clear, because NA/A input was stimulatory at low doses and inhibitory at high doses of estradiol. Further, it was proposed that GnRH pulse generator in OVX ewes is subserved by endogenous NA/A input and is therefore unresponsive to exogenous input.

***Growth and Attainment of Puberty***

The acquisition of reproductive competence, or puberty, in ewes and cows is characterized by the expression of behavioral estrus and the initiation of regular ovarian cycles. Endocrine regulation of puberty in heifers and ewes is centered around the inhibitory actions of estradiol on gonadotropin secretion and has been recently reviewed (Kinder *et al.*, 1987). Briefly, although shortly after birth, the ovary and pituitary are capable of responding to exogenous gonadotropins and GnRH, respectively, the occurrence of high frequency pulsatile LH secretion is suppressed in prepubertal ewes and heifers. According to the **gonadostat hypothesis,** systemic LH concentrations are suppressed prior to puberty by the strong negative feedback action of estradiol on the hypothalamic-pituitary axis. As the time of puberty approaches, sensitivity to the inhibitory action of the steroid decreases,

allowing a gradual increase in LH secretion which in turn stimulates follicular growth and development. As a result of follicular growth, estradiol secretion rises, subsequently stimulating the first preovulatory surge of gonadotropins and ovulation (for references, see Foster and Ryan, 1981; Kinder *et al.*, 1987). Although decreased sensitivity to estradiol negative feedback is associated with puberty, it is not entirely clear if this phenomenon is a consequence of puberty rather than the cause of it.

The mechanism(s) underlying the endocrine regulation of puberty appears to be quite complex and most probably involves the interaction of several different factors, including growth rate and seasonal photoperiodic cues. In prepubertal heifers and ewes, and other species, rate of body growth and development has been shown to exert a strong influence the timing and onset of puberty. Undernourished, growth retarded ewes and heifers attain puberty at a later age than their well-nourished counterparts (for references, see Kinder *et al.*, 1987). Although it is not too surprising that photoperiod influences the attainment of puberty in the ewe (for review, see Foster *et al.*,1986; Ebling and Foster, 1989; Yellon *et al.*, 1992), seasonal daylength cues also affect the age at puberty in heifers (Hansen *et al.*, 1983; Petitclerc *et al.*, 1983; Schillo *et al.*, 1983). In addition, photoperiod has been reported to affect body growth in both species (Forbes *et al.*, 1979; Peters *et al.*, 1980), and therefore it is possible that at least some of the effects of photoperiod on sexual maturation may be indirectly mediated through altered growth.

Ewe lambs that are born in the spring generally attain puberty in the autumn at about 30 wk of age; however, if lambs are born out of season, in the autumn, puberty is not attained at 30 wk and is delayed until the lambs are about 1 year of age, a time that corresponds to the autumn breeding season (Foster and Ryan, 1981). It was subsequently

determined that the ability to attain puberty under the inductive influence of short days relied upon previous exposure to long day photoperiods (Ebling and Foster, 1989). Further, Yellon and Foster (1985) determined that exposure of ewe lambs to as little as 1 wk of long days (15L:9D) from 21 to 22 wk of age, preceded and followed by short days (9L:15D), was sufficient to induce puberty in this species. The effects of photoperiod on the attainment of puberty, in ewe lambs, appear to be mediated through melatonin secretion because pinealectomy (Kennaway *et al.*, 1985) and denervation of the pineal gland (superior cervical ganglionectomy; Foster *et al.*, 1988) delayed the occurrence of puberty and the normal timing of puberty could be restored by administration of exogenous melatonin (Foster *et al.*, 1988). Collectively, these data suggest that attainment of puberty in ewe lambs is entrained to the subsequent breeding season by exposure to the long photoperiods of summer prior to the inductive short daylengths of autumn.

Early experiments investigating the effect of photoperiod on body growth of prepubertal heifers revealed that exposure to a total of 16 hr of light per day significantly increased body weight (12 kg increase) and average daily gain (10% increase) as compared to heifers exposed to 9 to 12 hr of light per day (Peters *et al.*, 1978). Similarly, Peters and coworkers (1980) reported that exposure of prepubertal heifers to 16L:8D increased average daily gain by 11 and 17 percent as compared to heifers exposed to natural photoperiod (9 to 12 hr light/day) or continuous light, respectively. Clearly these data suggest that body growth is enhanced in prepubertal heifers under conditions of increased photoperiod.

Season also appears to influence the age at onset of puberty because several investigators have reported that puberty occurred earlier in dairy heifers born in the spring than in those born in the autumn or winter (Hawk *et al.*, 1954; Menge *et al.*, 1960; Roy *et*

*al.*, 1980). More recent experiments, designed specifically to avoid the confounding of season of birth with season of puberty, suggest that heifers born in the fall attain puberty at a younger age than those born in the spring (Schillo *et al.*, 1982; 1983). The effects of season are presumably mediated through photoperiod, because heifers exposed to a long-day photoperiod (natural photoperiod of autumn and winter plus supplemental lighting; 16L:8D) had a tendency ($P \simeq .10$) to initiate estrous cycles earlier than heifers under natural photoperiod of autumn and winter (average 10.3 hr light/day) without supplemental lighting (Peters and Tucker, 1978).

Schillo and coworkers (1983) conducted an experiment to determine the stage of life at which season affects the age of puberty in heifers. In this study, Angus × Holstein heifers born in the fall and spring were maintained under natural photoperiods until 6 months of age, at which time they were moved to environmental chambers that simulated the seasonal changes in photoperiod and temperature characteristic of spring to fall (Sp-F) or fall to spring (F-Sp). Heifers born in the fall attained puberty at a younger age (307 vs 334 days) than those born in the spring, regardless of the photoperiod they were exposed to after 6 months of age. Additionally, fall- and spring-born heifers exposed to the Sp-F photoperiod, from 6 to 12 months of age, attained puberty earlier than those exposed to the F-Sp photoperiod. From these data it was suggested that the influence of season on the attainment of puberty in heifers may vary with age, because exposure to short photoperiods prior to 6 months of age (fall-born heifers) was associated with advanced onset of puberty, whereas, exposure to similar conditions (fall to spring photoperiod) after 6 months of age appeared to delay puberty. Further, the effects of season of birth on age at puberty may be mediated by changes in growth and(or) LH secretion, because average daily gain (from 6

to 9 months of age) and mean serum LH concentrations (from 6 to 7 months of age) were greater in fall- than in spring-born heifers.

Subsequent experiments investigating the effect of supplemental lighting on the age at puberty in heifers have suggested that this practice may be a useful management tool for cattle producers. Exposure to increased photoperiod (18L:6D) beginning at 22 or 24 wk of age reduced the age at first ovulation and estrus in heifers born from February to July, as compared with control heifers exposed to the prevailing natural photoperiod; however, mean weekly LH concentration was unaffected by supplemental lighting (Hansen *et al.*, 1983). Similarly, Petitclerc and coworkers (1983) reported that supplemental lighting (18L:6D) increased growth rate and feed efficiency and accelerated the onset of puberty in heifers maintained on either a low or high plane of nutrition.

Although the influence of photoperiod on the age at puberty is presumed to be mediated through nocturnal secretion of melatonin, few experiments have been conducted to investigate the effect of exogenous melatonin on the attainment of puberty in heifers. Tortonese and Inskeep (1992) reported that prepubertal beef heifers, born during late winter, receiving exogenous melatonin, in the form of a hydrogel-based implant, early in the summer for a period of 5 wk attained puberty at a younger age than did untreated control heifers. Further, the effect of melatonin on the onset of puberty did not appear to be mediated through body growth or nutritional factors, because body weight, average daily gain and body weight:age ratio at puberty did not differ between melatonin-treated and control heifers. Several experiments have suggested, that in sheep, continuous exposure to increased concentrations of melatonin from constant release implants is perceived as a short-day photoperiodic signal (Lincoln and Ebling, 1985; O'Callaghan *et al.*, 1991b). In light

of this finding and their results, Tortonese and Inskeep (1992) have suggested that, exposure to short days at a critical age (in this case, 3 to 4 months) may heighten the stimulatory effects of a subsequent long-day photoperiod (Schillo *et al.*, 1983; Hansen *et al.*, 1983) on the attainment of puberty in heifers, a hypothesis that is consistent with the notion of the cow as a long-day breeder (Peters and Riley, 1982; Hansen and Hauser, 1984).

The effect of season of birth on the age at puberty in cows has been suggested to provide a mechanism whereby photoperiod might concentrate calving dates to spring and summer (Schillo *et al.*, 1983). Fall-born heifers would reach puberty in the summer or early fall after birth and would tend to calve the subsequent spring and summer. Spring-born heifers would achieve puberty, at an older age, the following spring and summer and would subsequently calve at a similar time of the year. Again this hypothesis is supportive of the concept that the cow may intrinsically follow the reproductive patterns characteristic of a long-day breeder.

The mechanism underlying the seasonal effects of photoperiod on the attainment of puberty in heifers is not known, but may involve regulation of the sensitivity of the hypothalamic-pituitary axis to the negative effects of estradiol, because Hansen and coworkers (1982) reported that LH secretion in response to exogenous estradiol was greater in OVX heifers exposed to a long-day photoperiod (18L:6D) than in those exposed to the natural photoperiod of winter. Clearly, there is a paucity of information concerning the role of photoperiod in the attainment of puberty in cows and, therefore, considerable research is required if we are to fully understand how season influences sexual maturation in this species.

*Mammary Growth in Heifers*

In addition to the other reproductive processes influenced by season or photoperiod in cows, there is evidence to suggest that photoperiod may be involved in the regulation of mammary growth in heifers (Petitclerc *et al.*, 1985; Sanchez-Barcelo *et al.*, 1991). Growth of bovine mammary tissue is isometric (increases at the same rate as body weight) or allometric (accelerated growth rate) depending on age and reproductive state. Isometric growth occurs from birth to 3 months of age and after 10 months of age until the heifer becomes pregnant (Sinha and Tucker, 1969). In contrast, allometric, or accelerated, mammary growth occurs between 3 and 9 months of age and during gestation (Swanson and Poffenbarger, 1979). Allometric growth is thought to be induced by the combined actions of ovarian steroids, prolactin and growth hormone, although the process is not well understood (Tucker, 1981).

Exposure of prepubertal and postpubertal dairy heifers experiencing isometric mammary growth, to a long-day photoperiod (16L:8D; 139 days) significantly increased mammary parenchymal weight (g/100 kg body weight) and total parenchymal DNA (an index of cell numbers; mg/100 kg body weight), as compared with heifers exposed to a short-day photoperiod (8L:16D). However, photoperiod did not influence the total weight of the mammary gland (parenchymal + extraparenchymal connective tissue and fat) nor the concentration of DNA in parenchymal tissue. Further, extraparenchymal tissue weight was significantly lower in heifers exposed to the long-day photoperiod. From these data it was concluded that exposure of heifers to an extended photoperiod specifically stimulated the growth of mammary parenchymal tissue. Additionally, the ability of photoperiod to influence mammary growth in heifers may be restricted to those periods when the gland is

growing isometrically, because exposure of heifers to long- or short-day photoperiods during an established allometric growth phase failed to alter mammary growth parameters (Petitclerc *et al.*, 1984). Further support for this concept was provided by the report of Newbold and coworkers (1991) that exposure of pregnant Holstein heifers to either long- or short-day photoperiods failed to alter mammary development during pregnancy, a time when mammary growth is allometric.

Not surprisingly, the mechanism whereby photoperiod mediates its effects on bovine mammary development appears to involve the secretion of melatonin, because midday feeding of melatonin (4 mg/kg bodyweight) to dairy heifers exposed to a long-day photoperiod (16L:8D) reduced mammary growth as compared to control heifers fed vehicle (Sanchez-Barcelo *et al.*, 1991). The reduction in growth was manifested as a reduction in the number of mammary parenchymal cells and increase in intraparenchymal fat. Heifers receiving melatonin had reduced serum concentrations of prolactin and elevated serum melatonin concentrations that were similar in amplitude and duration to that which occurs under a short-day (8L:16D) photoperiod. From these data it was suggested that the influence of photoperiod on mammary growth may reflect an inhibitory effect of short-day photoperiod, rather than a stimulatory effect of long-day photoperiod as was reported previously (Petitclerc *et al.*, 1985). Further, it was proposed that the melatonin-induced reduction in mammary growth was mediated through an increase in fat accretion within the gland and(or) through reduced secretion of prolactin, a known mammogenic hormone. Interestingly, the authors failed to mention that the negative effects of melatonin were observed even though criteria previously used by this laboratory to determine the growth phase (isometric or allometric) of mammary tissue in heifers (Petitclerc *et al.*, 1985)

indicated, that at the start of the present experiment, the mammary gland was in an allometric growth phase.

Collectively, the data presented in this chapter clearly demonstrate that while our knowledge concerning the role of photoperiod and melatonin in the regulation of seasonal reproduction and attainment of puberty in sheep has flourished, little progress has been made toward determining the mechanism whereby melatonin regulates various reproductive events in cattle. Various experiments with cows and heifers have illustrated that season influences the return to estrus after calving and attainment of puberty and that administration of exogenous melatonin can alter the occurrence of these events. Importantly, although photoperiod has been shown to alter LH secretion in OVX heifers and cows and the significance of the role that this gonadotropin plays in the regulation of the postpartum interval and onset of puberty has been clearly demonstrated, it has been difficult to show an effect of season on postparturient and peripubertal LH secretion. Further, to date, only one experiment investigating the effect of exogenous melatonin on LH secretion in cows has emerged in the scientific literature and the results did not support an acute action of melatonin on LH secretion. In this experiment, daily administration of melatonin (15 mg, i.m. at 1600 hr) to estradiol-treated OVX beef heifers maintained under the increasing photoperiod of spring (March 19 to June 4) tended to increase mean weekly LH, but not FSH, concentrations ($P=.12$) but failed to alter pulse frequency, amplitude or duration of either gonadotropin (Critser *et al.*, 1987a) suggesting that melatonin may provoke a subtle change in the secretion of LH in OVX heifers.

Clearly, the potential effect of melatonin on LH secretion should not be dismissed based on the results of a single experiment. Additionally, the apparent lack of available

information concerning the effects of melatonin on LH secretion in cattle, in light of the observed photoperiod-induced alterations in ovarian function, stresses the need for further research in this area. It seems likely that a methodical investigation of the ability of the indoleamine to influence bovine gonadotropin secretion under a variety of physiological conditions and photoperiods will resolve the issue. Further, because melatonin has been observed to influence follicular steroidogenesis *in vitro*, the possibility, however unlikely, that some of the effects of photoperiod are mediated through a direct ovarian action of melatonin should not be ignored.

# EXPERIMENTS 1 AND 2: SECRETION OF LUTEINIZING HORMONE IN RESPONSE TO EXOGENOUS MELATONIN IN POSTPARTUM BEEF COWS SUBJECTED TO SHORT-TERM CALF REMOVAL AND IN OVARIECTOMIZED BEEF HEIFERS

## Introduction

Cows, specifically *Bos taurus*, are not considered to be seasonal breeders because ovulation and mating occur throughout the year. Nevertheless, there is evidence to suggest that photoperiod may influence reproductive processes in this species. Season of the year has been demonstrated to alter the pattern of LH secretion in ovariectomized dairy heifers (Critser *et al.*, 1983) and ovarian follicular development in beef heifers (McNatty *et al.*, 1984c). After parturition cows experience a period of anestrus characterized by the absence of regular ovarian cycles and reduced pituitary (Moss *et al.*, 1985) and systemic concentrations of LH (Humphrey *et al.*, 1983). Return to estrus after calving is associated with increased frequency of pulsatile secretion of LH that stimulates follicular development and ultimately ovulation (Walters *et al.*, 1982c). Although nutrition (Wiltbank *et al.*, 1962) and suckling (Short *et al.*, 1972) have been identified as factors that affect the interval from calving to first estrus, the effect of season on the length of the postpartum interval in cows is less well understood. Shorter postpartum intervals have been reported for beef cows calving in the summer or fall compared with winter or spring (Hansen and Hauser, 1983; King and Macleod, 1984). Beef cows exposed to supplemental lighting (18 hr light:6 hr dark) after calving in the fall and winter had shorter intervals from calving to first estrus compared with cows maintained under natural photoperiod (Hansen and Hauser, 1984).

Seasonal reproduction in domestic livestock and other mammals is believed to be mediated through changes in photoperiod, with some species responding to decreasing (short day breeders; sheep, goats) and others to increasing hours of daylight (long day breeders; horses, mink). It is generally accepted that the pineal gland mediates the photoperiodic control of seasonal reproduction through nocturnal secretion of melatonin (Reiter, 1980). In most mammalian species, including sheep (Rollag and Niswender, 1976) and cows (Hedlund *et al.*, 1977; Berthelot *et al.*, 1990), melatonin is secreted in a diurnal rhythm with maximal systemic concentrations occurring during the scotophase (dark period). In the ewe, seasonal alterations in the circadian pattern of melatonin secretion provide an endocrine code for daylength (Karsch *et al.*, 1991) that entrains an intrinsic circannual rhythm of reproduction (Woodfill *et al.*, 1991). Past photoperiodic history (direction of change in daylength) determines if the reproductive response to changing photoperiod is inductive or inhibitory (Robinson and Karsch, 1987) and different portions of the annual photoperiodic cycle play different roles in timing the onset and duration of the breeding season (Malpaux *et al.*, 1989; Wayne *et al.*, 1990). Melatonin, appears to control initiation and termination of the breeding season by modulating the ability of estradiol to reduce the frequency of LH pulses (Karsch and Moenter, 1990), presumably acting at the level of the hypothalamus to reduce the pulsatile release of GnRH (Karsch *et al.*, 1987).

Effect of melatonin on LH secretion in cattle is unclear. High affinity melatonin receptors have been identified in bovine hypothalamus (Cardinali *et al.*, 1979) and subcutaneous melatonin implants delayed the onset of estrus and ovulation in anestrous Shorthorn beef cows (Sharpe *et al.*, 1986), suggesting that this indoleamine may influence the interval from parturition to first estrus. Exposure of estradiol-treated ovariectomized

beef heifers to decreasing photoperiod increased serum LH concentrations (Critser *et al.*, 1987b), whereas administration of exogenous melatonin to similarly treated heifers under conditions of increasing photoperiod (Critser *et al.*, 1987a) had no significant effect on the gonadotropin.

In the present study, two experiments were conducted to evaluate the effect of constant release melatonin implants on induced LH secretion in postpartum (pp) beef cows and estradiol-treated ovariectomized heifers. To facilitate the detection of variations in LH secretion, cows were subjected to short-term calf removal because this procedure has been demonstrated to elevate systemic concentrations of LH (Edwards, 1985).

## Materials and Methods

**Experiment 1**. Beef cows were assigned to control (C; n=5) or treatment groups (MLT; n=7) at calving and received none or one melatonin (MLT; Sigma Chemical Co.) implant (3.35 mm i.d., 4.67 mm o.d. silastic tubing containing 250 mg melatonin) s.c. in each ear the day (d) after calving, respectively. Implants were constructed following the procedure of Moore (1981) and were soaked in saline (3 hr) prior to insertion. Calving occurred from May 17 to June 10. A weekly daytime (10 ml; d 7 to 35 pp) and one nighttime blood sample (10 ml; d 27 pp) were collected from each cow at 1000 and 2400 hr, respectively, and the sera were analyzed for MLT by radioimmunoassay (RIA). Additionally, after collection of each weekly blood sample, MLT-treated cows were examined to confirm the presence of implants. On d 27 pp, cows were fitted with jugular cannulas (46 cm of Intramedic polyethylene tubing, size PE 90; Clay Adams). Calves were separated (0800 hr) from their dams (d 28 pp) and blood was collected every 15 min for 4 hr (1000-1400 hr) on d 28, 29 and 31. During the 4 d of separation, calves were maintained in a facility out of sight and sound by the dams and were fed a milk replacer. After the 4 hr sampling period on d 31, each cow was injected (i.v.) with 100 $\mu$g GnRH (Cystorelin®, Sanofi Animal Health, Overland Park, KS) and sampling at 15 min intervals continued for 2.5 hr. Sera were analyzed for LH by RIA. Cows and calves were reunited on d 31 at the end of the last sampling period. Cows were checked twice daily for estrus with a vasectomized bull for 15 d beginning d 28 pp. Implants were removed from treated cows on d 38 pp.

**Experiment 2.** Beef heifers, ovariectomized (OVX) for 6 mo, were assigned randomly to C (n=4) or MLT (n=4) groups and received none or one MLT implant (1 g MLT in silastic tubing) s.c. in the neck (May 31). Weekly daytime blood samples (10 ml) were collected from the jugular vein on the side opposite the implant and analyzed for MLT. On day 29 after implant insertion, all heifers were fitted with jugular cannulae on the side opposite the implant and injected with estradiol-17$\beta$ ($E_2$; 1.5 mg/2 ml corn oil, i.m.). Blood was collected prior to and every 45 min after $E_2$ treatment for 24 hr and the sera analyzed for LH.

**Radioimmunoassays.** Blood samples were allowed to clot at room temperature and were then stored at 4° C for 24 hr. Sera were separated by centrifugation (500 × *g*) for 15 min at 4° C and stored at -20° C until assayed.

Serum LH was quantified by RIA following the method of McCarthy and Swanson (1976) with some modification. Purified bLH (USDA-bLH-B-5, AFP 5500) was iodinated by reacting the gonadotropin (5 $\mu$g/25 $\mu$l double-distilled $H_2O$) with [$^{125}$I]-sodium iodide (1 mCi; Amersham) and chloramine-T (10 $\mu$l; .5 mg/ml) for 1 min followed by the addition of sodium metabisulfite (10 $\mu$l; 1 mg/ml) to terminate the reaction. Radiolabeled LH was separated from free $^{125}$I by addition of the mixture to an anion exchange column (3 cc syringe with 2.54 cm of Tygon tubing attached to the hub) containing AG 2 × 8 resin (chloride form, 100-200 mesh; Biorad Labs) that had been sequentially rinsed with .5 M sodium phosphate buffer (PB; pH 7.6, 4-5 ml), .05 M PB-5% BSA (pH 7.5, 1 ml) and .05 M PB (pH 7.5, 4-5 ml) prior to use. After depositing the reaction mixture on the resin bed, the column was rinsed twice with .05 M PB (1 ml) and the eluate collected in a culture tube (12 × 75 mm; borosilicate glass) containing .01 M phosphate buffered saline (PBS)-1%

gelatin (pH 7.2, 1 ml; Knox gelatin). The tube containing the radiolabeled LH was capped, stored undiluted at 4°C and used without further purification.

The LH assay was validated using rabbit anti-bovine LH (PKC-242; 1:80,000) and sheep anti-rabbit gamma globulin (PKC-pool C; 1:60) as the primary and secondary antibodies, respectively. Recovery of LH standard (.125-2.0 ng/tube) added to 200 $\mu$l calf serum averaged 108 $\pm$ 3.9% and standard dilutions of serum (50-300 $\mu$l) from ovariectomized heifers were parallel to the standard curve. Assay sensitivity was .125 ng/tube ($P<.01$) and sample volume assayed was 200 $\mu$l per tube except after GnRH injection (50-100 $\mu$l serum/tube). Intra- and interassay coefficients of variation were 7.0 and 10.2%, respectively, for Exp. 1 and 9.4 and 9.7%, respectively, for Exp. 2. Cross-reactivity of the primary anti-serum with bFSH and bGH was .3 and 2.9%, respectively. Samples were assayed in duplicate and concentrations of LH are expressed as ng equivalents of NIH-LH-b10/ml serum.

Radioimmunoassay for melatonin was performed as described by Claypool *et al.* (1989) with minor modification, and validated for bovine serum. Serum (350 $\mu$l) was extracted with chloroform prior to assay and final determinations were corrected for procedural loss. Recovery of melatonin standard (5-50 pg) added to 300 $\mu$l serum averaged 96$\pm$2.4% and standard dilutions of serum (150-450 $\mu$l) were parallel to the standard curve. Extraction efficiency averaged 81.3$\pm$1.5% and sensitivity of the assay was .5 pg/tube ($P<.01$). Intra- and interassay coefficients of variation for two serum pools were 8.9 and 9.4%, and 6.5 and 8.1%, respectively. All samples were extracted and assayed in duplicate.

**Statistical Analysis**. Data on serum LH (from 0 to 240 min) over d 28, 29 and 31 and after GnRH (90 to 150 min) on d 31 pp from Exp. 1 and weekly daytime MLT levels

from both experiments were subjected to natural log (ln) transformation prior to analysis of variance (ANOVA) for repeated measures because visual inspection revealed that the standard errors (SE) were proportional to the means. For clarity, means of natural log data (M-ln) were back-transformed to familiar units for presentation. Due to the curvilinear nature of logarithmic functions, direct back-transformation of SE derived from log data may result in severe overestimation of the SE of the mean. Therefore, standard errors presented are approximate and were obtained using the following procedure: 1) standard errors of M-ln (SE-ln) were computed, in the usual manner for untransformed data (Snedecor and Cochran, 1980), using the appropriate error mean square from the ANOVA of ln transformed data, 2) upper (M-ln + SE-ln) and lower (M-ln - SE-ln) confidence boundaries of M-ln were computed and the resulting values back-transformed and 3) approximate SE were computed as one-half of the difference between the back-transformed upper and lower confidence boundaries [ $.5( e^{M\text{-}ln + SE\text{-}ln} - e^{M\text{-}ln - SE\text{-}ln} )$].

The ANOVA of LH data over the 3 d included treatment group (G), sampling day (D) and time (T; min), as well their interactions, and the F-statistic for each main effect was computed using animal within treatment group (A/G), A/G × D and A/G × T mean squares, respectively, as the error term. In the ANOVA of LH data after GnRH on d 31 (Exp. 1) and weekly melatonin data for both experiments, effect of treatment was tested using A/G mean squares, whereas effect of time after GnRH and G × T interaction and weekly MLT sampling day and G × D interaction were tested using A/G × T and A/G × D mean squares, respectively, as the error term. Differences in variability in LH secretion between control and treated cows were analyzed by F-test of variance (Snedecor and Cochran, 1980). Variances for the control and treatment groups across the 3 days and

within a sampling day were derived from the A/G and A/T x D mean squares, respectively. The A/G mean square from the ANOVA of LH data following GnRH on d 31 was used to derive the variances for control and treatment groups in response to the decapeptide. Differences in mean midnight melatonin concentration were analyzed by t-test for independent samples. Because, total sample size in Exp. 1 was less than 20 (Snedecor and Cochran, 1980) estrous data were subjected to Fisher's Exact test (Bailey, 1981) rather than chi-square analysis. Serum LH concentrations in Exp. 2 were analyzed by ANOVA for repeated measures on nontransformed data. Differences among means were tested for significance by Fisher's Protected Least Significant Difference (FPLSD) test (Petersen, 1985).

In Exp.1, several control (2) and treated (3) cows were missing either one (three cows: 2 MLT and 1 C), or two (two cows: 1 MLT and 1 C) weekly daytime melatonin samples. However, because ANOVA for repeated measures of weekly melatonin concentrations from cows with complete data sets (seven cows: 4 MLT and 3 C) revealed no treatment × day interaction ($P=.13$), missing sample values for each of the affected cows were estimated by the arithmetic mean of the remaining observed values for that cow. The subsequent data were subjected to ANOVA; however, 7 degrees of freedom were subtracted from the within and total degrees of freedom and consequently, the error mean square adjusted to correct for estimation of the missing samples. In addition, during the last week of blood sampling (d 28 to 35) it was discovered that two cows were missing one ear implant and one cow both implants. Data from these cows were included in all analyses because ANOVA (treatment classes: MLT-kept, MLT-lost and C; $P<.01$) of weekly daytime MLT levels (uncorrected for missing sample values) and subsequent separation of

the means using FPLSD indicated that daytime serum levels of MLT (pg/ml) in the three cows that lost implants were not lower than those in cows retaining their implants over the duration of the experiment ($26.3 \pm 4.8$ vs $33.5 \pm 5.0$; $P > .05$) and were greater than those in control cows receiving no implants ($16.4 \pm 2.3$; $P < .05$).

## Results and Discussion

Mean weekly daytime MLT levels (pg/ml) were significantly greater ($P<.01$) in cows ($29.8\pm3.4$) and OVX heifers ($34.4\pm6.0$) receiving MLT implants compared to control cows ($16.6\pm2.2$) and OVX heifers ($12.9\pm2.2$). In contrast, average midnight concentrations of melatonin (pg/ml) did not differ ($P>.05$) between C ($118.2\pm25.0$) and MLT ($141.9\pm19.6$) groups in Exp. 1 and may be the result of considerable animal-to-animal variation in the secretion of endogenous MLT as reflected in the large standard errors associated with the means. Significant animal-to-animal variation in melatonin secretion has been reported for the ewe (English *et al.*, 1987) and rapid fluctuations in nocturnal melatonin secretion were observed in cyclic dairy cows near the time of the summer solstice (Berthelot *et al.*, 1990). Similar to this study, feeding prepubertal heifers melatonin significantly elevated MLT levels during light but not dark periods compared with untreated control heifers (Sanchez-Barcelo *et al.*, 1991).

Secretory patterns of LH for control and treatment groups on d 28, 29 and 31 of Exp. 1 are depicted in Figures 1, 2 and 3, respectively. One cow in the control group was excluded from the LH analysis over the 3 d, but not after GnRH on d 31, because blood samples were not collected on d 29. Mean LH secretion over the 3 d did not differ (treatment $\times$ day interaction; $P>.05$) between the MLT and C groups; however, daily LH secretion during the 4 hr bleeding window was more variable among C than MLT cows ($P = .002$). A similar trend in the variation of LH secretion was observed following GnRH injection on d 31 pp. In contrast, no difference in the variability of LH secretion among C and MLT cows was detected across the 3 sampling days ($P>.05$). It is possible that the

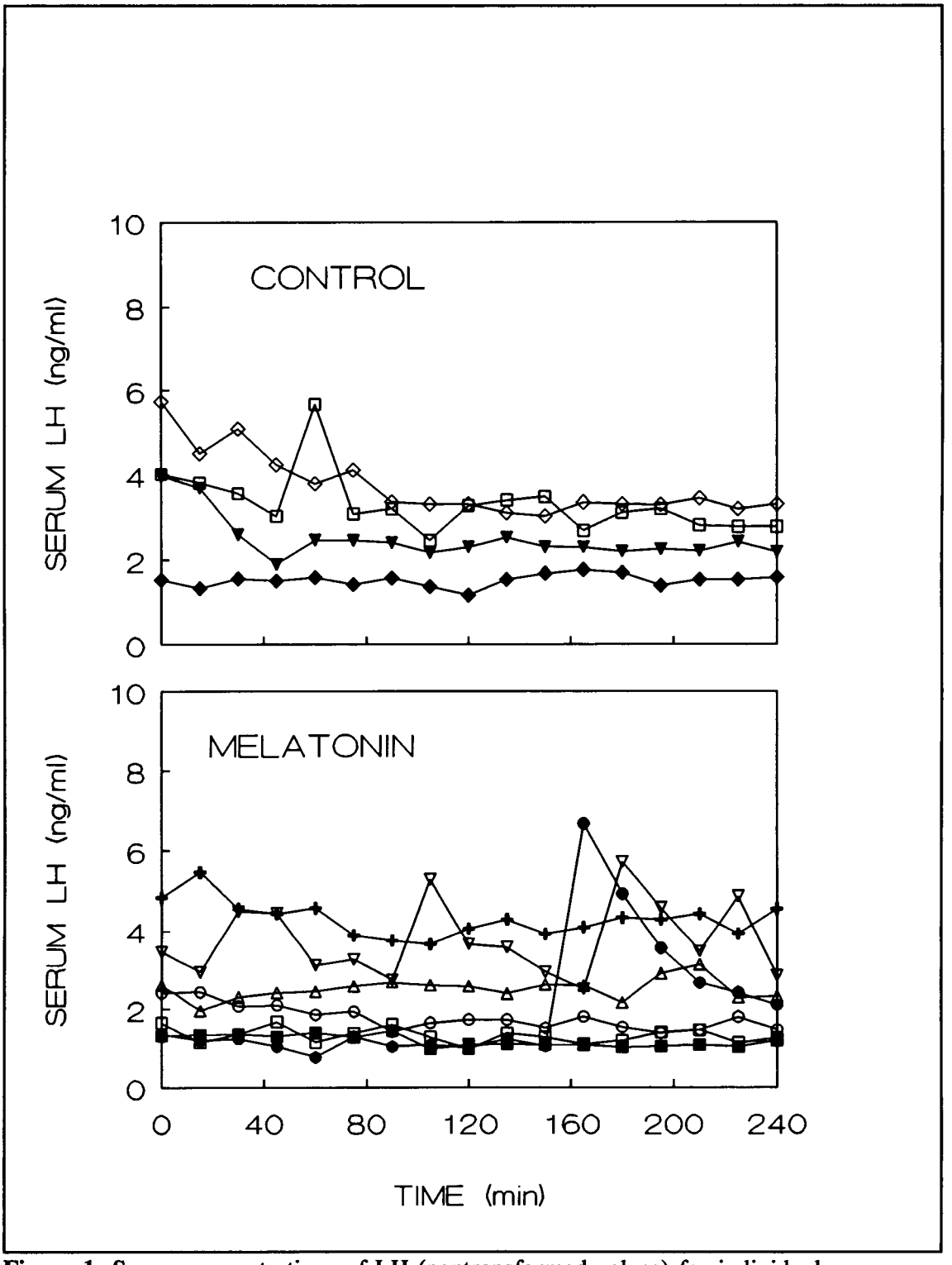


**Figure 1.** Serum concentrations of LH (nontransformed values) for individual cows within a treatment group on day 28 pp after CR. Symbols representing individual cows are consistently represented in Figures 1-4.

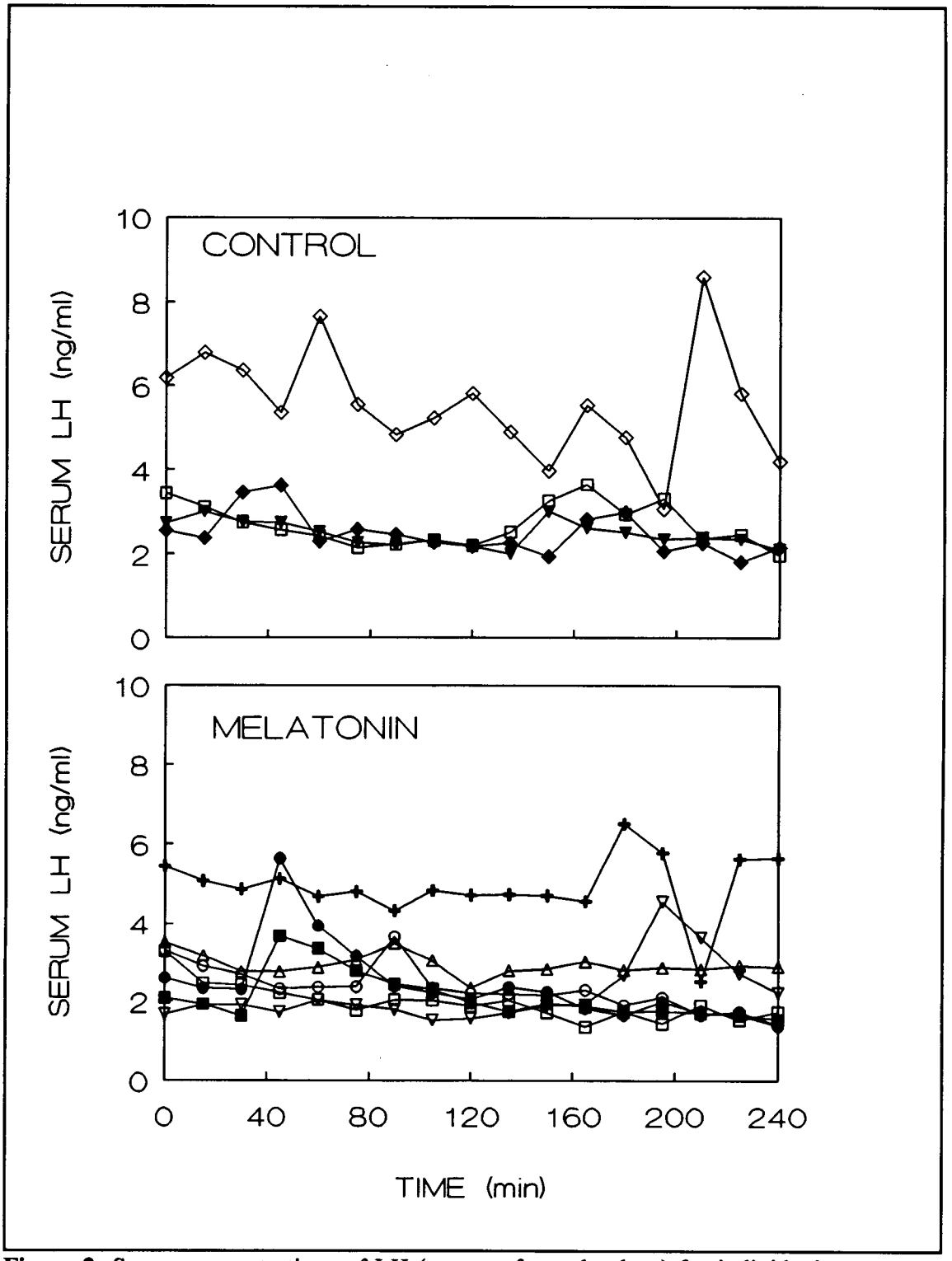


**Figure 2.** Serum concentrations of LH (nontransformed values) for individual cows within a treatment group on day 29 pp after CR.

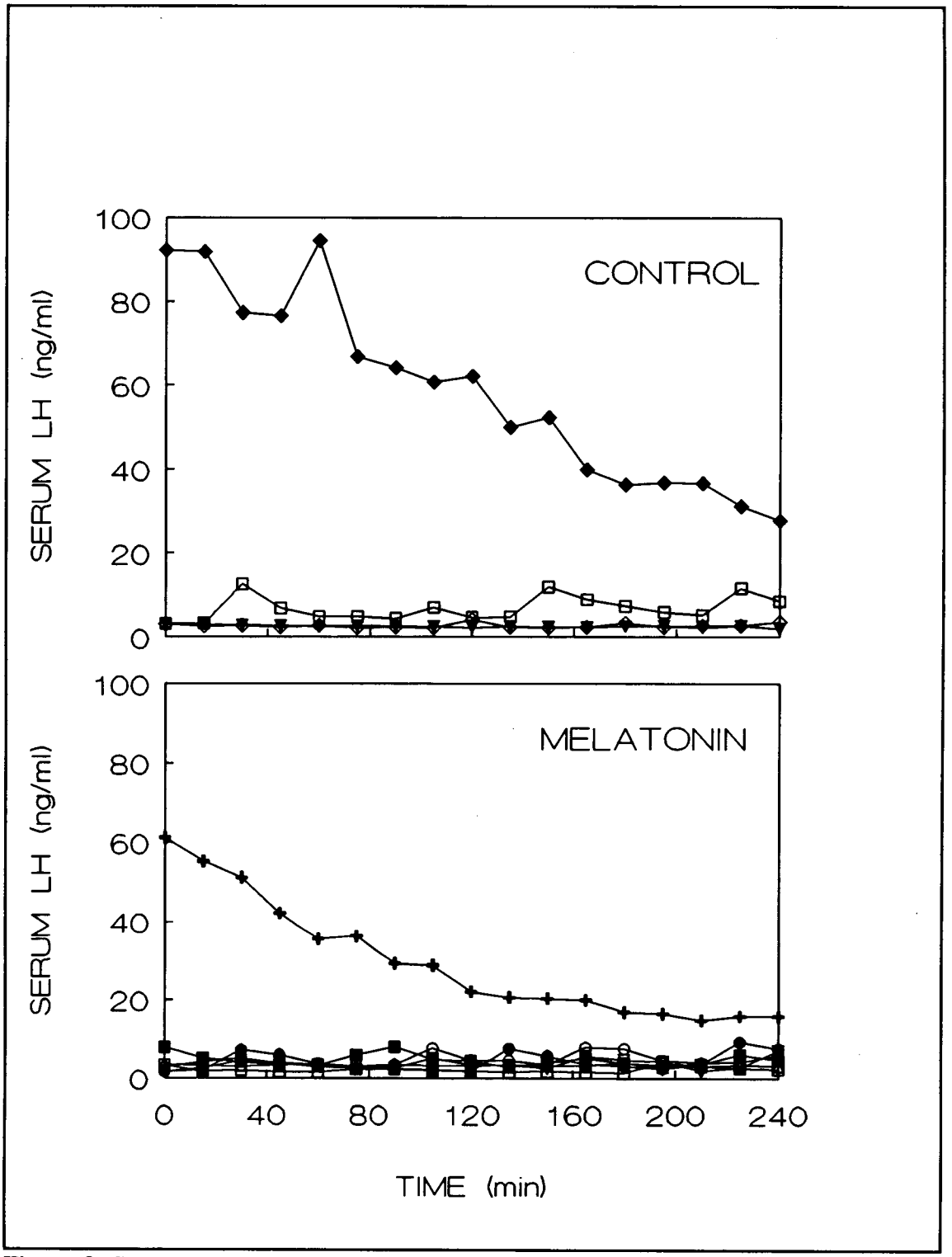


**Figure 3.** Serum concentrations of LH (nontransformed values) for individual cows within a treatment group on day 31 pp after CR.

failure to detect a significant alteration in LH concentration between the two groups may have occurred because the sampling interval and(or) frequency were not sufficient to enable the detection of discrete pulses of LH in all animals. Although, treatment was without effect, serum LH concentration did differ significantly among sampling days ($P=.02$). Mean LH (ng/ml) was greater on d 31 ($5.3\pm1.1$) as compared to d 28 ($2.3\pm.5$; $P<.01$) and 29 ($2.7\pm.5$; $P<.05$) pp. The observed increase on d 31 appeared to be the result of one cow in each group that had greatly elevated LH concentrations on that day. Short-term calf removal (CR; Edwards, 1985) and early weaning of cows (Walters *et al.*, 1982c) have been reported to increase circulating levels of LH.

All animals responded to GnRH treatment with an increase in LH (Figure 4) but because there was extreme animal-to-animal variation during the first 90 min after GnRH treatment, only data from 90 to 150 min were analyzed. Secretion of LH in response to GnRH did not differ between treatment groups ($P>.10$); however, the response to GnRH was more variable in C than MLT cows ($P<.05$). Collectively, these data suggest that although elevated daytime concentrations of MLT did not alter mean basal or GnRH-induced levels of LH, the indoleamine may have reduced the variability in basal and GnRH-induced LH secretion after CR. Reduced variability in tonic or low amplitude LH secretion could possibly influence the initiation of estrus after calving because return to estrus and ovulation is associated with an increase in basal levels and pulse frequency of LH (Walters *et al.*, 1982c). Animals with "smoother" patterns of LH release, *e.g.*, fewer pulses or fluctuations, might exhibit longer pp intervals. Administration of MLT implants to postpartum Shorthorn beef cows delayed the return to estrus by 10 d compared with untreated control cows (Sharpe *et al.*, 1986).

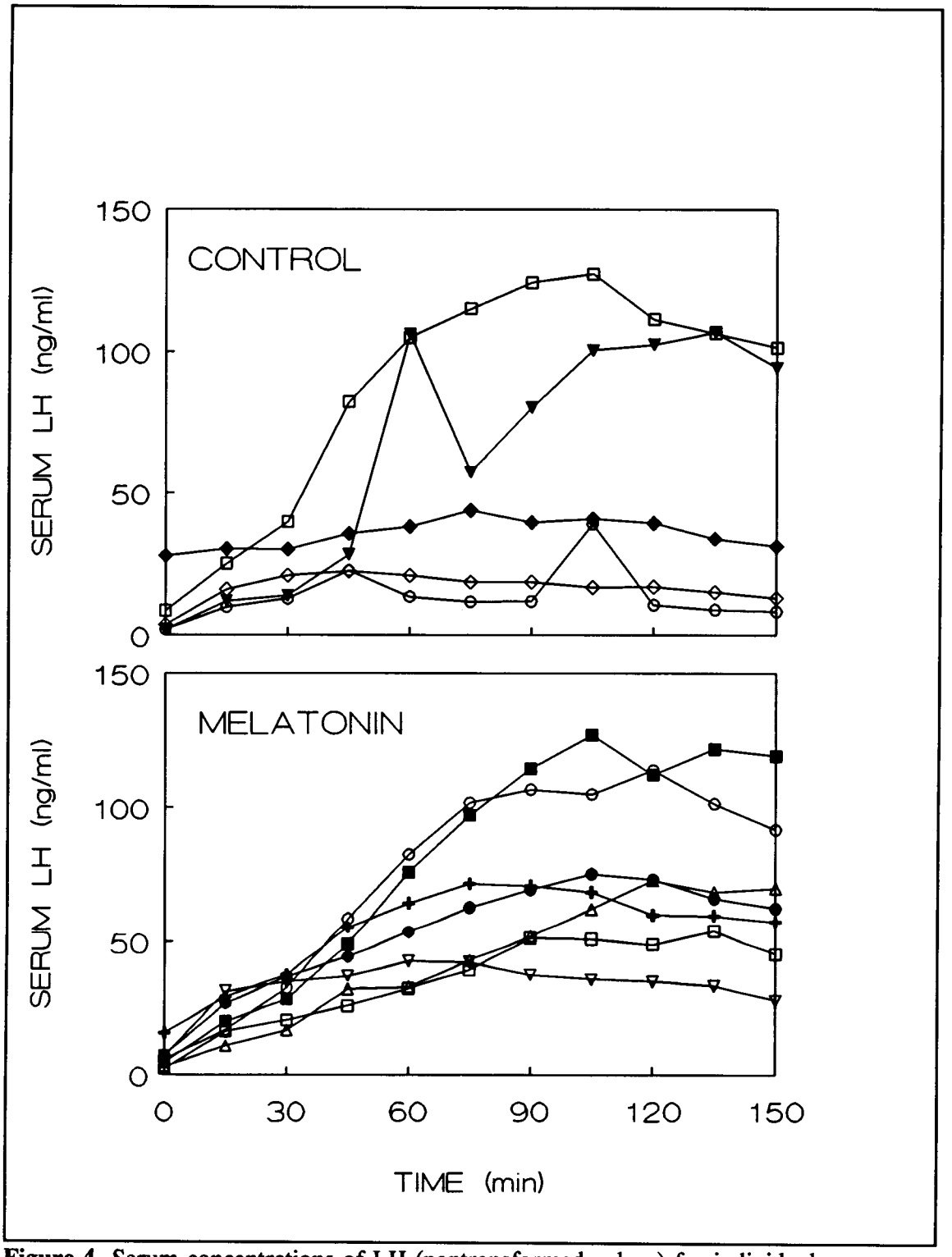


**Figure 4.** Serum concentrations of LH (nontransformed values) for individual cows within a treatment group after GnRH injection (100 $\mu$g) on day 31 pp. GnRH was injected i.v. at time 0.

In the present experiment, fewer MLT cows (0/7) returned to estrus within 15 d of calf removal compared with C (3/5; P=.04) and rectal palpation of cows after CR revealed that one cow in each group had ovulated without exhibiting estrus. Exposing suckled cows to 18 hr light/day (18L:6D) commencing at calving in the autumn, significantly decreased the interval from calving to estrus as compared with cows under the natural autumn photoperiod (short days); however, this artificial extension of the natural photoperiod had no effect on LH secretion (Hansen and Hauser, 1984). In light of these latter data and the finding that constant release melatonin implants can convey a short day photoperiodic signal in ewes (O'Callaghan *et al.*, 1991b), it is tempting to suggest that elevated daytime levels of MLT may have been perceived as a photoperiodic cue for short days in this experiment, resulting in fewer MLT cows returning to estrus after calf removal. Because reduced variability in LH levels among MLT-treated cows was associated with absence of estrus and ovulation after CR, it is conceivable that MLT may have prevented these events through subtle modulation of LH secretion.

Alternatively, it is possible that melatonin may have delayed the return to estrus in response to CR by impairing ovarian function; perhaps indirectly through alteration of FSH secretion and(or) a direct follicular action of the indole. Follicle stimulating hormone is requisite for follicular development (Richards, 1980) and thus a reduction in FSH release could have a negative impact on this process. However, because basal and estradiol-induced secretion of FSH in suckled postpartum beef cows was not influenced by photoperiod (Hansen and Hauser, 1984) and daily injection of melatonin increased secretion of the gonadotropin in estradiol-treated OVX heifers maintained under increasing photoperiod

(Critser *et al.*, 1987a) a significant reduction in FSH secretion in response to melatonin in the present experiment seems unlikely.

There is a growing body of evidence to support the concept that melatonin may also influence reproductive events through direct action at the gonads. Melatonin binding sites have been demonstrated in the ovaries of hamsters, rats and humans (Cohen *et al.*, 1978), but not sheep (Helliwell and Williams, 1992). *In vivo* administration of melatonin reduced testis weights in rats (Debeljuk *et al.*, 1971) and stimulated luteal progesterone secretion in monkeys (Webley and Hearn, 1987). The effect of melatonin on follicular steroidogenesis appears to be confined to progesterone synthesis in granulosa cells, because the indoleamine increased progesterone, but not estradiol, secretion by rat (Fiske *et al.*, 1984), human (Webley and Luck, 1986; Brzezinski *et al.*, 1992), bovine (Webley and Luck, 1986) and ovine (Baratta and Tamanini, 1992) granulosa cells *in vitro*. Season had no effect on residual aromatase activity in or LH-induced androstenedione secretion from granulosa cells or theca interna *in vitro*, respectively, nor did it influence the diameter or numbers of granulosa cells in small (2 to 4.5 mm), intermediate (5 to 7.5) and large ($\geq$ 8 mm) follicles or follicular fluid concentration of estradiol in dominant follicles of beef cows (McNatty *et al.*, 1984c). Further, patterns of follicular atresia in the autumn and winter did not differ from those observed in the spring (McNatty *et al.*, 1984c). In light of these findings, it is difficult to envisage a mechanism whereby melatonin might impair folliculogenesis through perturbation of the steroidogenic pathway in bovine granulosa cells and(or) theca interna.

Melatonin is thought to modulate LH secretion in the ewe at the level of the hypothalamus with no direct effect on the pituitary (Robinson *et al.*, 1986), and presumably

this is the case with the cow, because in this study MLT treatment had no effect on pituitary response to GnRH.

In Exp. 2, administration of $E_2$ to ovariectomized (OVX) heifers resulted in a biphasic pattern of LH release (decrease followed by increase) in all heifers regardless of treatment group (Figure 5) suggesting that systemic concentrations of $E_2$ were increased as a result of the injection. Beck and Convey (1977) reported that LH secretion decreased from 2 to 6 hr and then increased reaching a peak at 18.5 hr after administration of $E_2$ implants to OVX heifers; LH levels appeared to return to baseline by 26 hr after treatment. In contrast, although the pattern of LH secretion in the two groups in the present study was similar during the negative feedback phase, LH levels gradually increased without reaching a peak during the sampling period. Differences in LH response between the two studies may reflect differences in rates of absorption of the steroid into systemic circulation. Heifers in the present experiment were extremely obese and thus it is possible that a significant portion of the $E_2$ injected was retained in the underlying fat, resulting in a somewhat attenuated LH response to the steroid.

Elevation of daytime serum MLT concentration for 4 wk failed to alter LH secretion ($P>.05$) in response to $E_2$ injection from that of control heifers. Similarly, daily administration of MLT (1500 hr) for 12 wk under conditions of increasing photoperiod (March to June) did not alter LH secretion ($P>.10$) at 4, 8 or 12 wk after MLT treatment in OVX beef heifers treated for 6 mo with $E_2$ implants (Critser *et al.*, 1987a). Collectively, these data suggest that increased levels of MLT, in the presence of elevated concentrations of $E_2$, have no effect on the secretion of LH. A possible explanation for this lack of effect is that the steroid treatment may have desensitized the hypothalamus to the action of

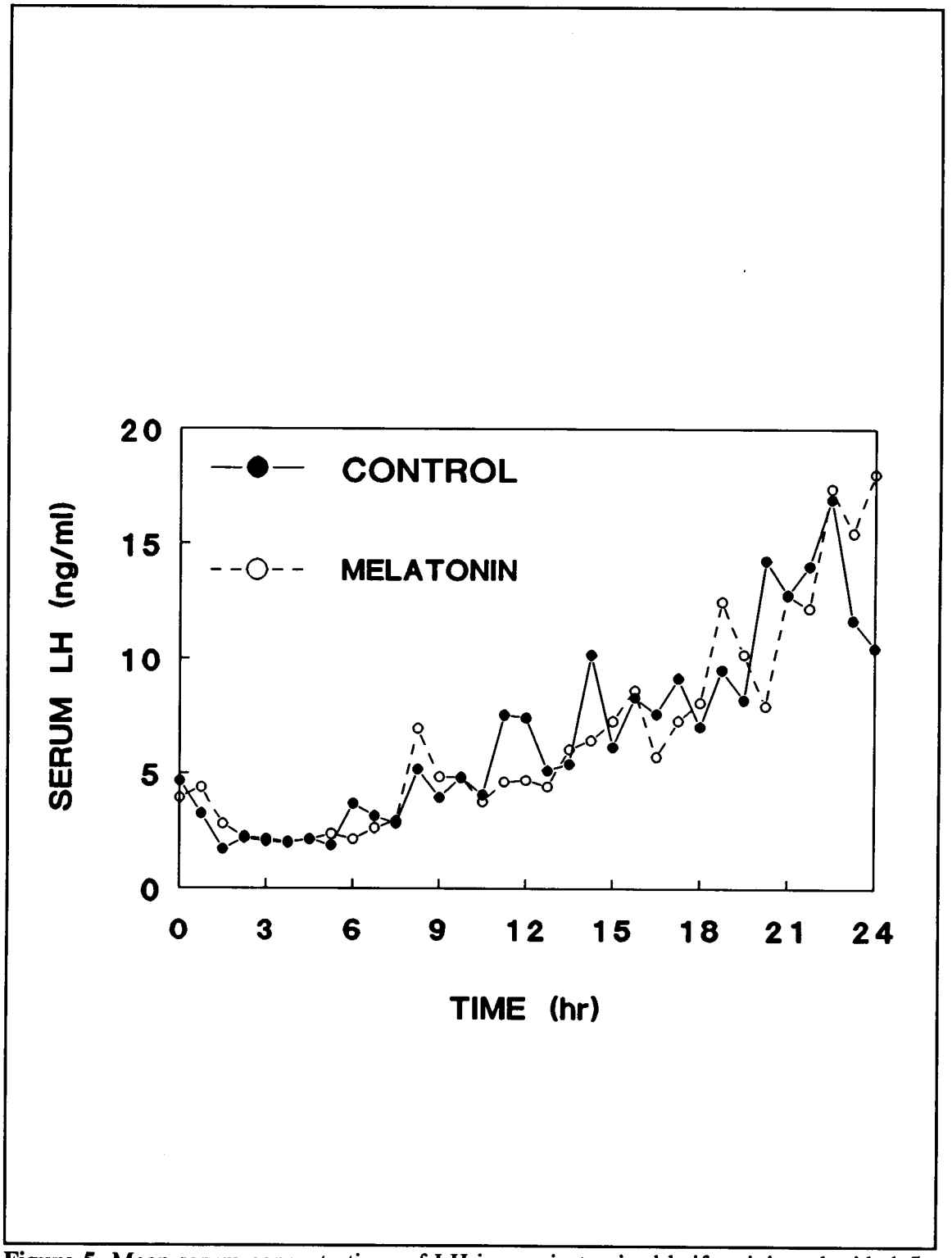


**Figure 5.** Mean serum concentrations of LH in ovariectomized heifers injected with 1.5 mg $E_2$ i.m. at time 0 (pooled SE=1.06).

melatonin. However, this latter possibility seems unlikely because $E_2$-treated OVX heifers under conditions of the natural photoperiod of fall to spring had significantly greater basal secretion of LH and increased LH pulse amplitude compared with similarly treated heifers exposed to extra light to simulate the photoperiod of spring to fall (Critser *et al.*, 1987b). Perhaps the ability of exogenous MLT treatment to alter LH secretion in $E_2$-treated OVX heifers is dependent upon the prevailing photoperiod. Should the constant release of melatonin be perceived as a short day cue, conditions of this study might be similar to those described previously (Critser *et al.*, 1987a) because a short day melatonin cue was imposed on OVX heifers during increasing photoperiod and did not alter LH secretion in response to $E_2$.

# Summary

Data from Exp. 1 demonstrate that exposure of late-spring calving beef cows to melatonin implants, beginning on day 1 postpartum, significantly increased daytime systemic concentrations of melatonin and reduced the variability in LH secretion after short-term calf removal. Further, reduced variability in LH levels among melatonin-treated cows was associated with the absence of estrus and ovulation after calf removal suggesting that melatonin may have prevented these events through subtle modulation of LH release. In addition, the finding that none of the melatonin-treated cows had returned to estrus by the end of the experiment is consistent with the hypothesis that constant release melatonin implants convey a short day photoperiodic cue (O'Callaghan *et al.*, 1991b) and that the return to estrus is delayed in cows calving under short photoperiods (Hansen and Hauser, 1984). Because the results of this experiment provide further evidence to support the concept that increased daily exposure to elevated systemic concentrations of melatonin impairs reproductive function in postpartum cows, a second experiment is warranted to confirm and extend the present findings.

With respect to Exp. 2, because the pattern of LH release in response to estradiol was not entirely consistent with previous studies (Beck and Convey, 1977), it is possible that the duration of the sampling period was not sufficient to allow the detection of an alteration in the interval to or amplitude and duration of the LH peak arising from the positive feedback effects of estradiol. Therefore, conducting a second experiment with leaner heifers, in conjunction with a slightly longer sampling period, might yield more promising results.

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# APPENDIX

# Gonadotropin-Releasing Hormone-Induced Secretion of Luteinizing Hormone in Postpartum Beef Heifers Maintained on Two Planes of Nutrition Before and After Breeding

## Abstract

An experiment was conducted to examine the effect of pre- and post-breeding nutrition on GnRH-induced LH release in beef heifers on d 3 and 14 of the postpartum period. Treatment groups consisted of heifers fed high (H; n=12) and low (L; n=12) planes of nutrition prior to breeding. Each group was further subdivided to receive either high or low planes of nutrition after breeding in a 2 × 2 factorial arrangement of treatments (H-H, H-L, L-H and L-L). On d 3 and 14 postpartum, heifers were injected with 100 $\mu$g GnRH (i.v.) and blood was collected *via* jugular venipuncture for LH analysis at 15 min intervals for 2.5 h and at 30 min intervals for an additional 2.5 h. Heifers fed a high level of nutrition post-breeding (H-H and L-H) released a greater mean quantity of LH (ng LH $\cdot$ $ml^{-1}$ $\cdot$ h) in response to GnRH on d 3 than did those fed a lower level of nutrition ($P < .05$). On d 14, mean quantity of LH released by the H-H group was greater than that of the other three groups ($P < .05$). These data indicate that post-breeding nutritional status significantly influenced pituitary responsiveness to GnRH on d 3 and that response to the decapeptide on d 14 was greatly enhanced by maintaining heifers on a high plane of nutrition both prior to and after breeding. In addition, the negative effect of low pre-breeding nutrition on GnRH-induced LH secretion on d 14 was not overcome by increasing the level of nutrition after breeding.

# Introduction

Factors such as nutrition (Randel, 1990), suckling (Williams, 1990) and season of calving (Hansen and Hauser, 1983; King and Macleod, 1984) influence the duration of postpartum anestrus in beef cows. Restricting total energy (Wiltbank *et al.*, 1962) or crude protein (Sasser *et al.*, 1988) prior to calving reduced the occurrence of estrus, prolonged the interval from calving to first estrus and reduced conception rate in beef cows. The mechanism by which undernutrition impairs postpartum reproductive function likely involves the regulation of LH secretion. Feeding beef cows a diet deficient in crude protein prior to parturition reduced pituitary content of gonadotropin and responsiveness to exogenous GnRH (Nolan *et al.*, 1988). Similarly, reducing dietary energy during gestation decreased pituitary response to estradiol (Echternkamp *et al.*, 1982) and GnRH (Killen *et al.*, 1989) in postpartum heifers.

Previous research investigating the effect of diet on reproductive performance and gonadotropin secretion in postpartum cows concentrated on feeding various levels of nutrition before and(or) after parturition, resulting in a paucity of information concerning the influence of nutritional status prior to pregnancy on reproductive function after calving. Therefore, the objective of this experiment was to determine the effect of pre- and post-breeding nutrition on GnRH-induced LH release in beef heifers on d 3 and 14 postpartum.

## Materials and Methods

***Animals and Experimental Diets.*** Hereford × Angus heifers of comparable age and weight were assigned, at weaning (October 10) to one of two treatment groups. Initial treatment groups consisted of heifers fed high (H; n=12) and low (L; n=12) planes of nutrition prior to breeding. Each group was further subdivided, after breeding (May 15 to August 1), to receive either high or low planes of nutrition in a 2 × 2 factorial arrangement of treatments (H-H, H-L, L-H, and L-L). Throughout the experiment all heifers had *ad libitum* access to rake-bunched hay and meadow aftermath daily or rangeland pastures with supplement provided at communal feeders that provided ample room for all heifers in a group to feed simultaneously. From weaning to parturition, heifers were periodically weighed and scored for body condition (BCS; scoring 1 to 9 with 1 = thin, 9 = obese) and supplement levels were adjusted as necessary in order to attain desired target weights at the first- and second-year breeding periods. Initial H and L target weights represented 60 and 65% of eventual mature weight of the cow, whereas L-L, L-H, H-L and H-H treatment groups were expected to range from 75 (L-L) to 90% (H-H) of eventual mature weight after calving. In this particular herd, mature BW is approximately 454 kg at a BCS of 5. Body weights were recorded at start of the experiment (November 7), breeding (May 13), mid-winter pre-calving (January 21) and within 24 h of calving (February 19 to March 16).

At weaning, heifers were gradually acclimated to protein supplementation by feeding increasing concentrations of barley and biuret for approximately 30 d, until supplement levels reached 1.35 kg barley and .05 kg biuret per heifer at which time heifers were separated into H and L treatment groups. Pre-breeding diets consisted of 1.40 kg (L) and

2.25 kg (H) total barley and biuret supplement • head$^{-1}$ • d$^{-1}$ in addition to rake-bunched hay and meadow aftermath. Heifers were fed to achieve target weights of 272 to 295 kg (L) and 295 to 319 kg (H) by the time of breeding.

At breeding, all heifers were exposed to Hereford × Angus bulls (one bull per 21 heifers) on 200 acre ranges from May 15 to August 1. During this period, all heifers received grass only with no additional supplementation. After breeding, the pre-breeding treatment groups were further divided into high and low post-breeding groups (n = 6 per group) and heifers on a low nutritional plane (L-L and H-L) continued to receive no supplementation whereas heifers on a high nutritional plane (H-H and L-H) received .9 kg barley and .04 kg biuret. After parturition, supplement level in the H-H and L-H groups was increased to 1.35 kg barley and .05 kg biuret to compensate for lactational demands. Level of supplementation in H-H and L-H heifers was adjusted as needed to keep condition scores at 5 to 6 and to attain target weights of 431 to 498 kg for H-H heifers by the second-year breeding. It was anticipated that the L-H and L-L heifers receiving no supplement would achieve weights of 340 to 386 kg over the same time period.

Heifers and calves were brought in from pasture within 24 h after parturition and on d 13 after calving. On d 3 and 14 postpartum, dams were separated from their calves and restrained in squeeze chutes. On both days, all heifers were injected (i.v.) with 100 $\mu$g GnRH (Cystorelin®, Sanofi Animal Health, Overland Park, KS) and blood samples (10 ml) were collected *via* jugular venipuncture for LH analysis at 15-min intervals beginning 30 min prior to and for 2.5 h after GnRH. At 2.5 h post-GnRH, samples were collected at 30-min intervals for an additional 2.5 h. Following the sampling period, cow and calf were reunited and returned to pasture.

***Radioimmunoassay.*** Blood samples were allowed to clot at room temperature and were then stored for 24 h at 4°C. Sera were separated by centrifugation (500 × *g*) for 15 min at room temperature and stored at -20°C until assayed for LH.

Serum LH was quantified by RIA following the method of McCarthy and Swanson (1976) with some modification. Purified bLH (USDA-bLH-B-5, AFP 5500) was iodinated by reacting the gonadotropin (5 μg/25 μl double-distilled $H_2O$) with [$^{125}$I]-sodium iodide (1 mCi; Amersham) and chloramine-T (10 μl; .5 mg/ml) for 1 min followed by the addition of sodium metabisulfite (10 μl; 1 mg/ml) to terminate the reaction. Radiolabeled LH was separated from free $^{125}$I by addition of the mixture to an anion exchange column (3 cc syringe with 2.54 cm of Tygon tubing attached to the hub) containing AG 2 × 8 resin (chloride form, 100-200 mesh; Biorad Labs) that had been sequentially rinsed with .5 M sodium phosphate buffer (PB; pH 7.6, 4-5 ml), .05 M PB-5% BSA (pH 7.5, 1 ml) and .05 M PB (pH 7.5, 4-5 ml) prior to use. After depositing the reaction mixture on the resin bed, the column was rinsed twice with .05 M PB (1 ml) and the eluate collected in a culture tube (12 × 75 mm; borosilicate glass) containing .01 M PBS-1% gelatin (pH 7.2, 1 ml; Knox gelatin). The tube containing the radiolabeled LH was capped, stored undiluted at 4°C and used without further purification.

The LH assay was validated using rabbit anti-bovine LH (PKC-242; 1:80,000) and sheep anti-rabbit gamma globulin (PKC-pool C; 1:60) as the primary and secondary antibodies, respectively. Recovery of LH standard (.125 to 2.0 ng/tube) added to 200 μl calf serum averaged 108 ± 3.9% and standard dilutions of serum (50 to 300 μl) from ovariectomized heifers were parallel to the standard curve. Assay sensitivity was .125 ng/tube ($P < .01$) and sample volume assayed was 200 μl per tube except after GnRH

injection (50 to 100 $\mu$l serum/tube). Intra- and interassay coefficients of variation were 8.6 and 8.2%, respectively (n = 6 assays). Cross-reactivity of the primary anti-serum with bFSH and bGH was .3 and 2.9%, respectively. Samples were assayed in duplicate and concentrations of LH are expressed as ng equivalents of NIH-bLH-b10/ml serum.

***Statistical Analysis.*** Differences in BW and BCS between the treatment groups were analyzed by ANOVA (Snedecor and Cochran, 1980) and differences among individual group means were tested for significance by Fisher's Protected Least Significant difference (FPLSD) test. Basal LH secretion was determined for individual heifers by calculating the mean LH concentration from samples collected 30 min, 15 min and immediately prior to injection of GnRH. After subtraction of basal LH level from each LH value, area under the LH response curve was determined for each heifer on both days by integration (summation of the area of trapezoids). Because there was extreme animal-to-animal variation in LH response during the final 90 minutes of the sampling period, only LH data from 0 to 210 min were integrated. The resultant areas (ng LH $\cdot$ $ml^{-1}$ $\cdot$ 210 min) for each day (d 3 and 14 postpartum) were subjected to ANOVA in which pre-and post-breeding diet were the main factors. To simplify presentation, mean area under the LH response curve was normalized to one hour (ng LH $\cdot$ $ml^{-1}$ $\cdot$ h) for all treatment groups.

## Results and Discussion

Mean BW did not differ among H and L treatment groups at the start of the experiment (184 ± 5 vs 183 ± 4 kg; $P > .05$); however, by the beginning of the breeding period the desired target BW had been attained and heifers receiving a high plane of nutrition were, on average, 24 kg heavier than those receiving a diet low in energy (298 ± 7 vs 274 ± 8 kg; $P < .05$). Pre-breeding level of nutrition also affected body condition at breeding. Heifers in the H treatment group had higher BCS than those in the L treatment group (5.5 ± .2 vs 5.1 ± .1; $P < .05$). Differences in mean BW after breeding and the subsequent split into post-breeding H and L nutrition groups are shown in Figure 1. Heifers in the H-H group were heavier during the mid-winter of their pregnancy and at calving (422 ± 6 and 398 ± 11 kg), respectively, than those in the H-L (386 ± 13; $P < .05$ and 339 ± 11 kg; $P < .01$) and L-L (372 ± 7 and 335 ± 6 kg; $P < .001$) groups. Body condition scores 2 mo after the end of the breeding period (October 1) did not differ among the four treatment groups ($P > .05$; mean = 5.7 ± .1); however, although not significant statistically ($P = .09$), heifers in the H-H group tended to have higher BCS (5 ± 0) than those in the H-L (4 ± .1) and LL (4.2 ± .1) groups after calving (May 5). Further, heifers receiving a high plane of nutrition post-breeding (H-H and L-H) were heavier at mid-winter (409 ± 9 vs 379 ± 7 kg; $P < .05$) and calving (337 ± 6 vs 384 ± 12 kg; $P < .01$) and on average had greater BCS late spring after calving (4.8 ± .3 vs 4.1 ± .3; $P < .05$) compared with those receiving a low plane of nutrition after breeding (H-L and L-L). Overall, BW increased from weaning to late gestation and decreased at calving in all heifers; however, BW of heifers in the H-L and L-L groups were consistently lower than those in

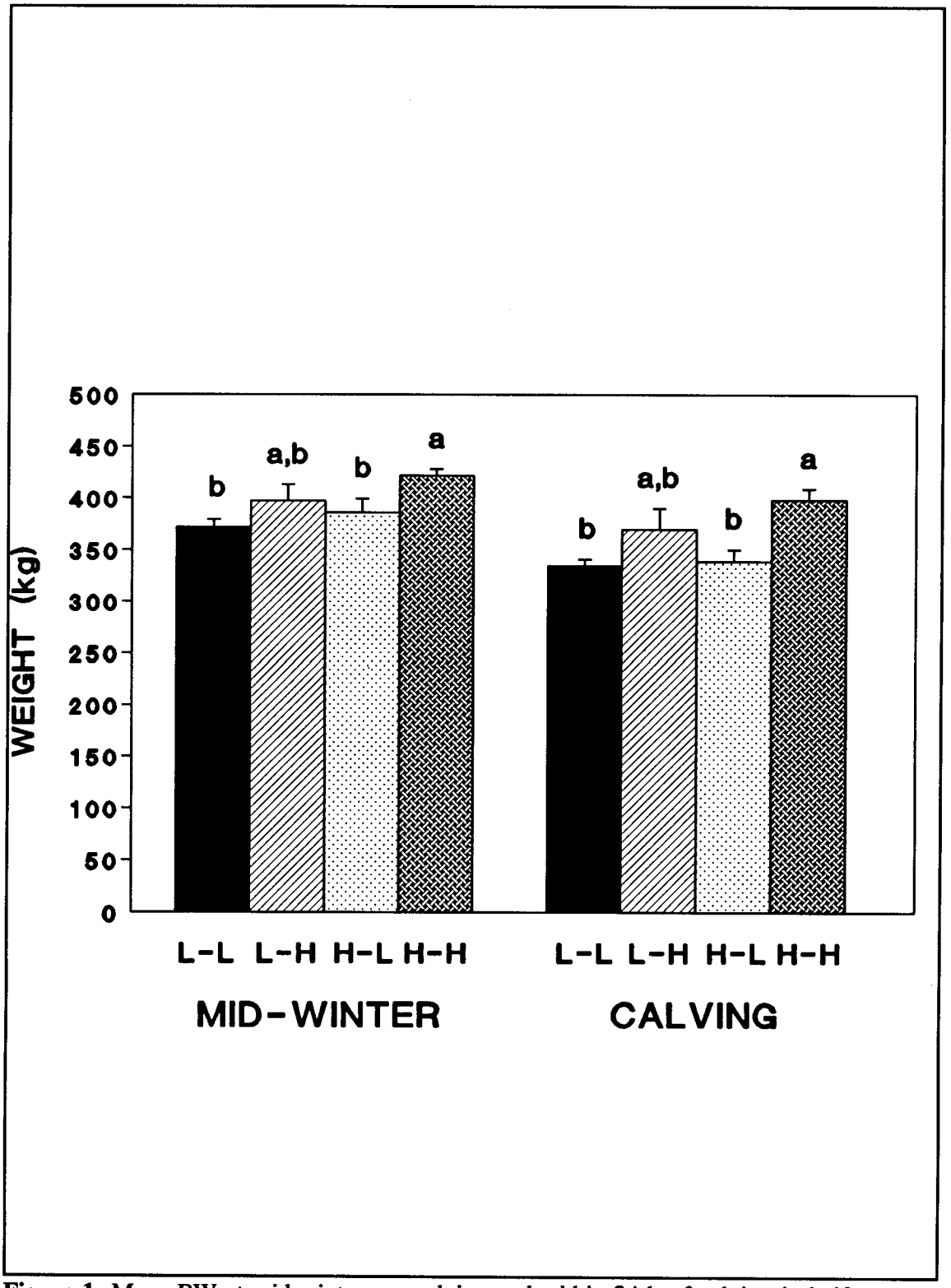


**Figure 1.** Mean BW at mid winter pre-calving and within 24 h of calving in heifers maintained on high (H) or low (L) planes of nutrition before and after breeding. Means (a,b) within a weighing date without a common superscript differ ($P < .05$).

the H-H and L-H groups.

Serum LH profiles for the four treatment groups on d 3 and 14 postpartum are depicted in Figures 2 and 3, respectively. All heifers, on both days, responded to GnRH injection with increased secretion of LH that approached or returned to basal levels by the end of the 5.5 h sampling period. Basal levels of LH did not differ significantly among treatment groups or between sampling days and averaged 3.6 $\pm$ .1 ng/ml. Others have reported no effect of pre- or postpartum nutrition on basal LH secretion in intact postpartum cows (Echternkamp *et al.*, 1982; Nolan *et al.*, 1988; Killen *et al.*, 1989). Post-breeding, but not pre-breeding, level of nutrition significantly altered pituitary response to GnRH on d 3 postpartum. As shown in Figure 4, heifers maintained on a high plane of nutrition after breeding (H-H and L-H ) released a greater mean quantity of LH ($P < .05$) than those fed a lower (H-L and L-L) level of nutrition (456 $\pm$ 39 vs 301 $\pm$ 39 ng LH $\cdot$ $ml^{-1}$ $\cdot$ h).

Results of this experiment indicate that both pre- and post-breeding level of nutrition influence pituitary response to GnRH in first-calf heifers during the early stages of the postpartum interval. Feeding heifers a low plane of nutrition post-breeding significantly reduced the amount of LH released in response to GnRH on d 3 postpartum. Similarly, secretion of LH in response to exogenous estradiol on d 14 and 28 postpartum was lower in heifers maintained on a low plane of nutrition beginning the last trimester of pregnancy (Echternkamp *et al.*, 1982) and the quantity of LH released after injection of GnRH between d 8 and 21 after calving was 50% lower in heifers nutritionally restricted during the final two trimesters of gestation (Killen *et al.*, 1989). Collectively these data demonstrate that feeding low levels of nutrition after breeding and during pregnancy is sufficient to suppress the ability of the pituitary to respond to exogenous hormonal stimuli during the early stages

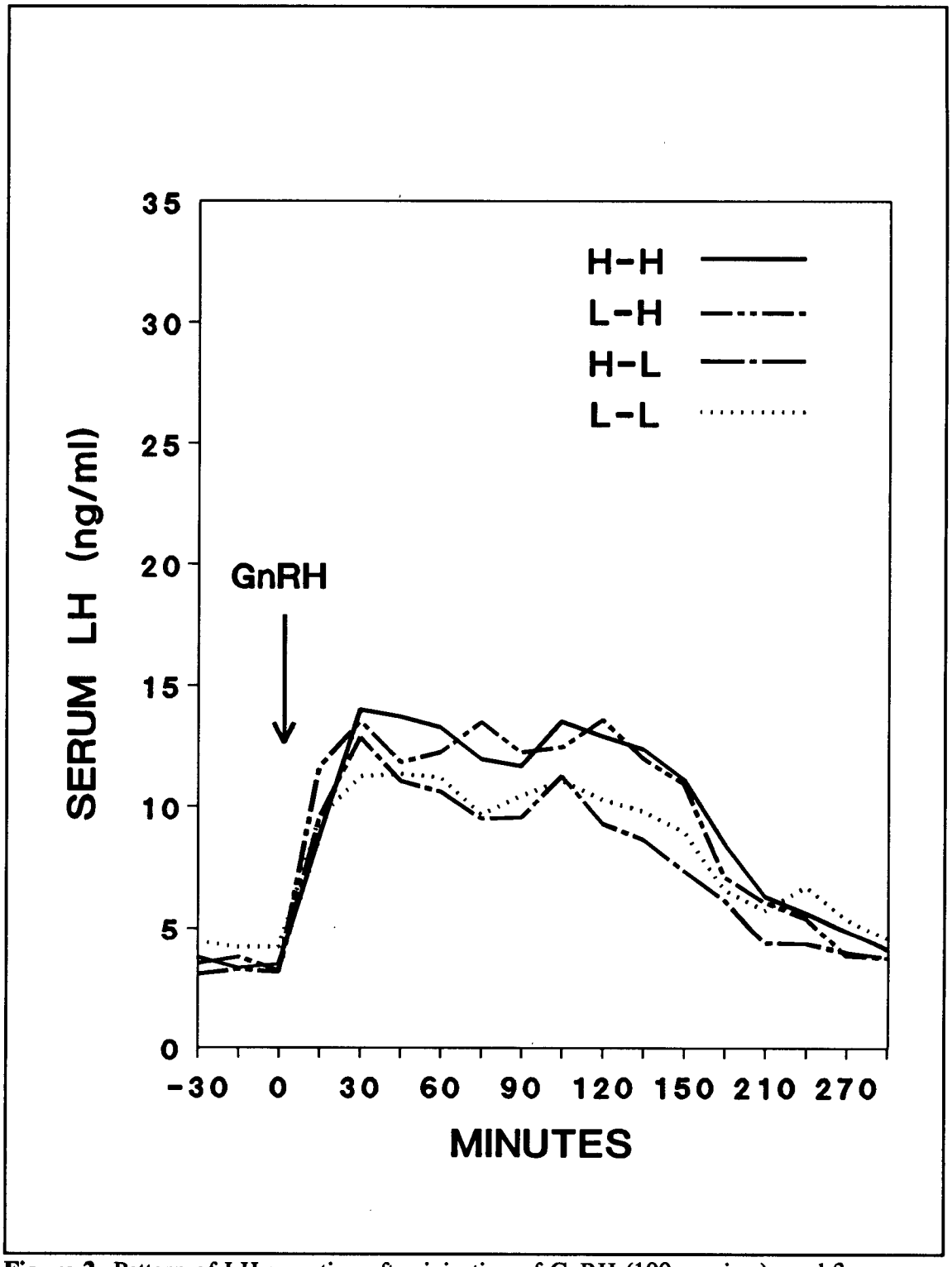


**Figure 2.** Pattern of LH secretion after injection of GnRH (100 $\mu$g, i.v.) on d 3 postpartum in heifers maintained on high (H) or low (L) planes of nutrition before and after breeding.

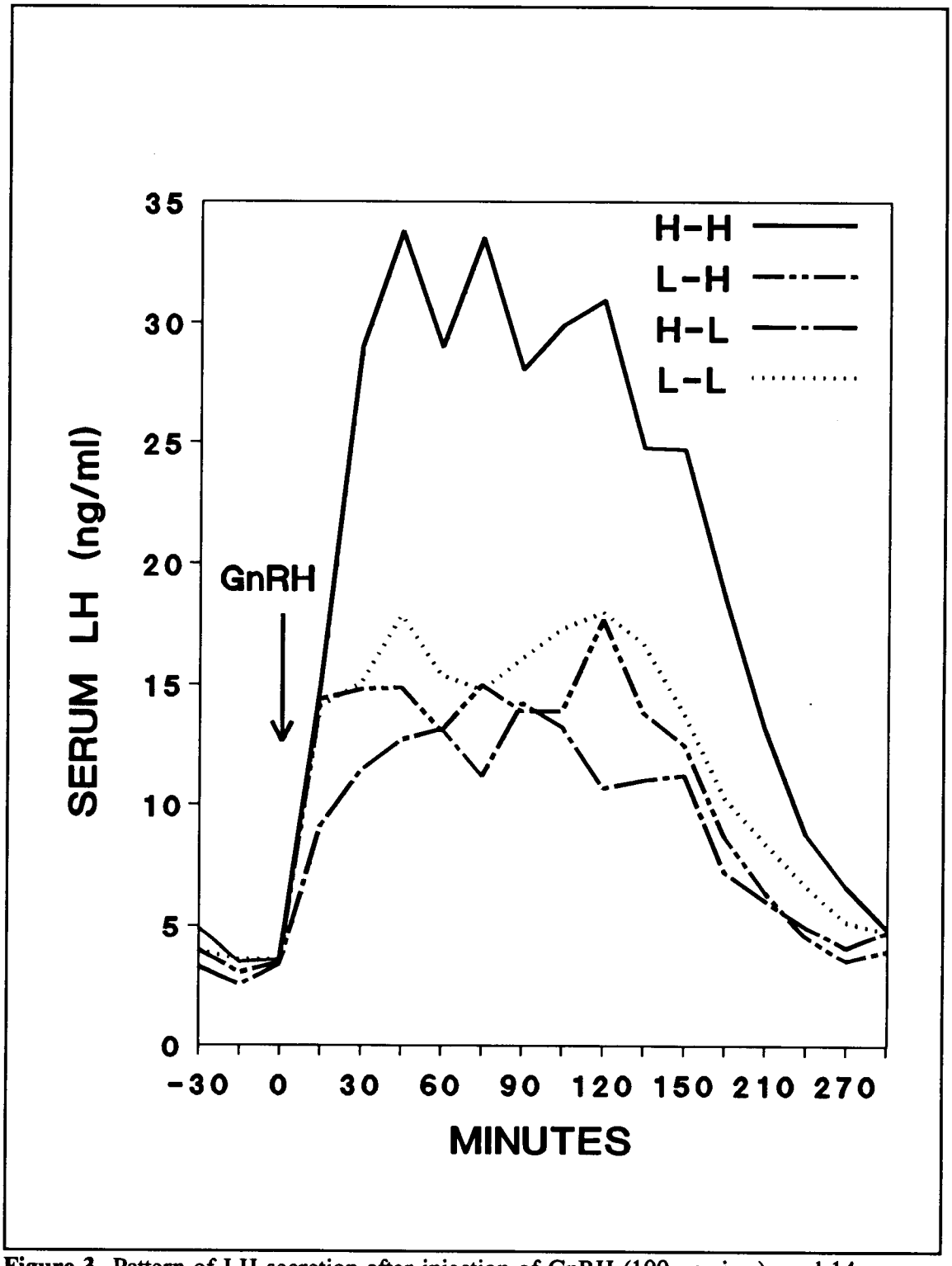


**Figure 3.** Pattern of LH secretion after injection of GnRH (100 $\mu$g, i.v.) on d 14 postpartum in heifers maintained on high (H) or low (L) planes of nutrition before and after breeding.

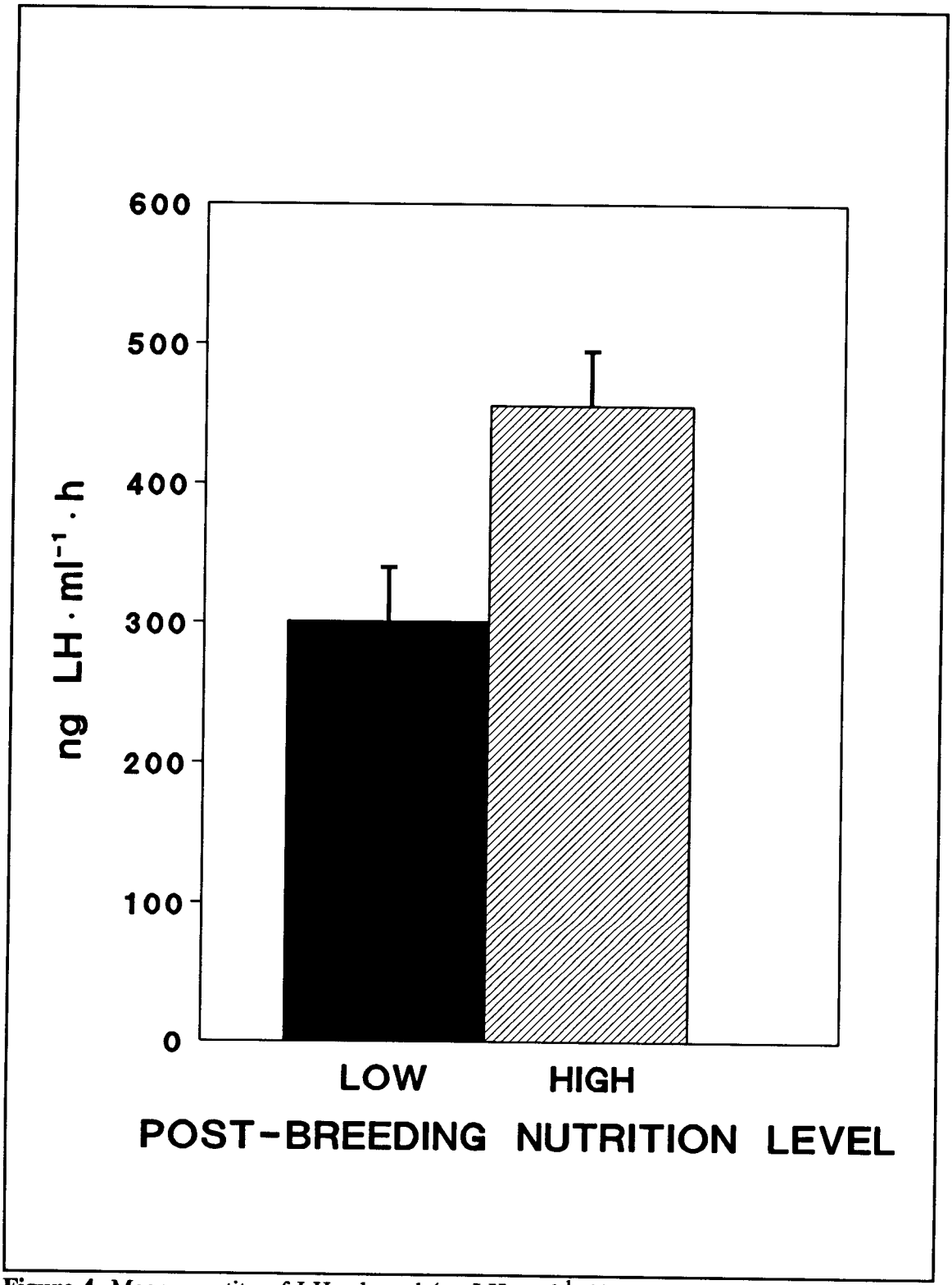


**Figure 4.** Mean quantity of LH released (ng LH · $ml^{-1}$· h) after injection of GnRH on d 3 postpartum in heifers maintained on high or low planes of nutrition after breeding.

of the postpartum period.

In contrast to the significant effect of post-breeding diet on GnRH-induced LH secretion that was observed on d 3, response to GnRH on d 14 (Figure 3) could not be attributed to the action of either pre- or post-breeding planes of nutrition alone and therefore most probably resulted from a combination of the two factors as suggested by the significant pre-breeding × post-breeding diet interaction from the ANOVA. Maintaining heifers on a high plane of nutrition both before and after breeding profoundly increased ($P < .05$) the quantity of LH released (ng LH $\cdot$ $ml^{-1}$ $\cdot$ h) after GnRH on d 14 (H-H; 1197 $\pm$ 193) as compared with heifers in the H-L (436 $\pm$ 193), L-H (544 $\pm$ 193) and L-L (622 $\pm$ 193) groups (Figure 5). In contrast, feeding a low plane of nutrition either prior to or after breeding reduced pituitary response to GnRH irrespective of the level of nutrition that preceded or followed it. These data suggest that on day 14 postpartum, the negative effect of feeding a low plane of nutrition prior to breeding could not be overcome by feeding a higher level of nutrition after breeding and that any benefit from feeding a high level of nutrition prior to breeding is subsequently suppressed by feeding a low plane of nutrition after breeding.

The differential effects of pre- and post-breeding nutrition on LH release in response to GnRH between d 3 and 14 is not readily explainable but may be related to the metabolic demands of lactation. Reduced response to GnRH in heifers in the L-L and H-L groups on d 14 was not unexpected because these animals received no dietary compensation for lactation and had the lowest BCS several months after calving. On the other hand, reduced pituitary response in the L-H group was somewhat surprising because these heifers received additional supplement to meet lactational demands and mean BCS after calving did not differ

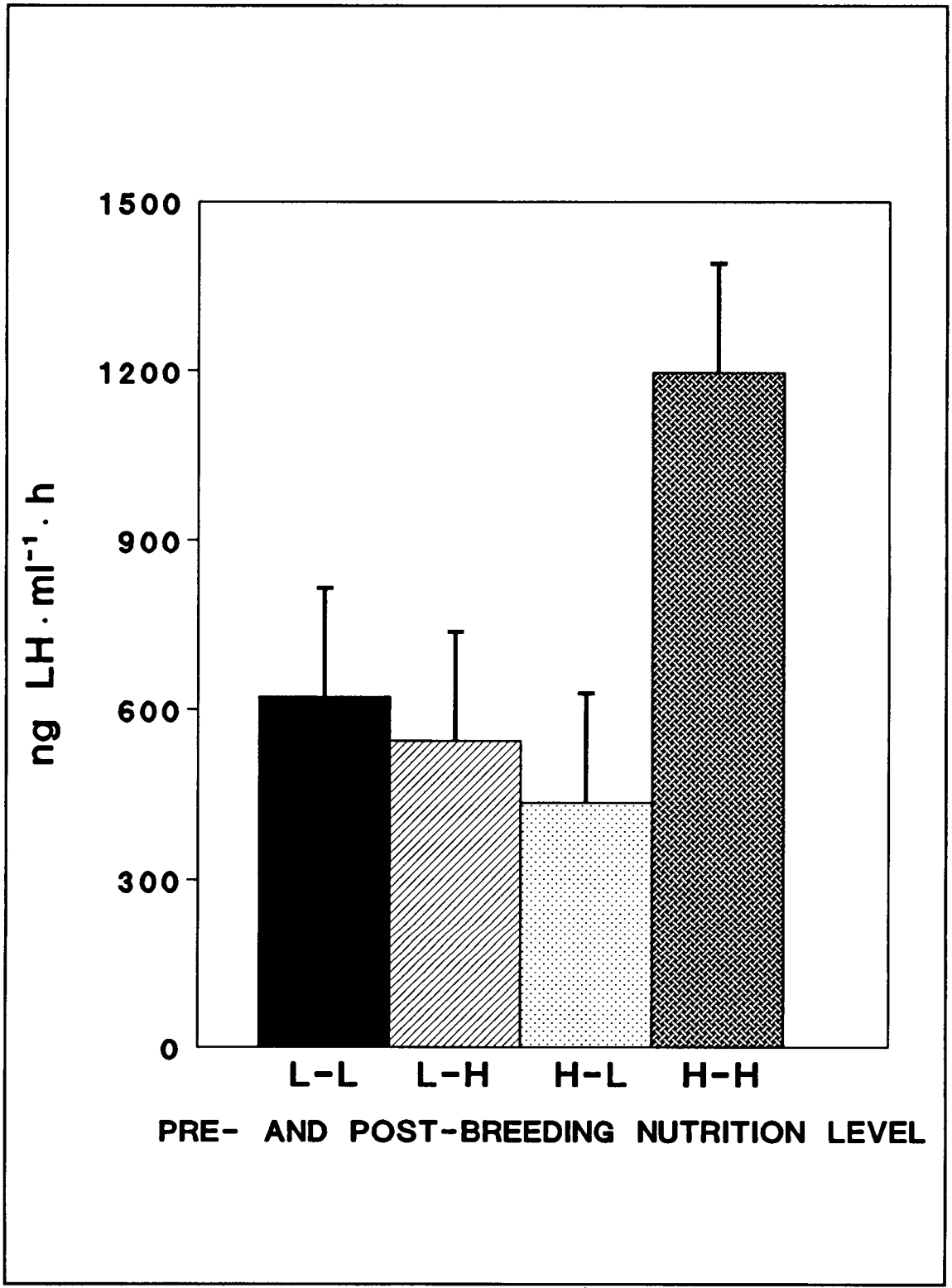


**Figure 5.** Mean quantity of LH released (ng LH · $ml^{-1}$ · h) after injection of GnRH on d 14 in heifers maintained on high (H) or low (L) planes of nutrition before and after breeding.

significantly from that of the H-H group suggesting that these heifers were not nutritionally stressed after calving. Hall and coworkers (1991) reported that systemic LH concentrations in suckled beef heifers maintained on low energy diet after calving failed to increase with increasing days postpartum as was observed in heifers receiving a high energy diet. Alternatively, differences between GnRH-induced LH secretion on d 3 and 14 may be due to differences in the physiological state of the hypothalamic-pituitary axis, perhaps in response to changes in steroidal milieu on the two days.

Early after parturition, secretion of LH in beef cows is characterized by low basal systemic concentrations and infrequent low-amplitude pulses of gonadotropin that gradually increase over the duration of the postpartum interval and eventually culminate in ovulation and the return of regular ovarian cycles (Arije *et al.*, 1974; Humphrey *et al.*, 1983; Nett, 1987). Suppression of LH release during late pregnancy and following calving is believed to result primarily from a reduction in pituitary stores of gonadotropin during late pregnancy (Rahe *et al.*, 1988) that persists for several weeks after parturition (Moss *et al.*, 1985; Nett *et al.*, 1988), but may also involve reduced secretion of GnRH from the hypothalamus (Allrich *et al.*, 1985; Leshin *et al.*, 1992) or reduced pituitary sensitivity to the decapeptide (Nett *et al.*, 1988). Elevated systemic concentrations of gonadal steroids during the latter stages of pregnancy and first week after parturition (Arije *et al.*, 1974; Humphrey *et al.*, 1983) are believed to inhibit LH synthesis, thus depleting pituitary content of the gonadotropin (Nett, 1987).

The precise mechanism whereby nutrition alters reproductive function in postpartum cows is not known; however, increasing evidence suggests the effects of undernutrition may be mediated at the level of the hypothalamic-pituitary axis to suppress LH

secretion (Schillo, 1992) and data from the present study are consistent with this hypothesis. Reduced pituitary sensitivity to exogenous estradiol or GnRH after calving in nutrient restricted heifers and cows appears to result from a decrease in the releasable pool of LH in the anterior pituitary (Echternkamp *et al.*, 1982; Nolan *et al.*, 1988; Killen *et al.*, 1989) but not from a reduction in the number of receptors for GnRH (Nolan *et al.*, 1988). Restricted dietary energy also negatively influenced content of GnRH in the preoptic area of beef cows (Connor *et al.*, 1990). Cows receiving a low energy diet prepartum followed by a high energy diet after calving (LE-HE) had less GnRH in the preoptic area on d 30 postpartum than did cows maintained on a low energy diet pre- and postpartum (LE-LE) or maintenance energy diet prepartum followed by high energy diet postpartum (ME-HE). In the present study, heifers in the L-H group released significantly less LH in response to GnRH on d 14 than those in H-H group and slightly less, although not significantly so, than heifers in the L-L group making it tempting to speculate that exposure to a low level of nutrition prior to breeding followed by a high level of nutrition after breeding might reduce the releasable pool of LH by reducing hypothalamic content of GnRH.

In conclusion, data presented here suggest that pre- and post-breeding nutrition influence pituitary sensitivity to GnRH early in the postpartum period of beef heifers. Pituitary sensitivity to exogenous GnRH on d 3 was altered by the effects of the post-breeding diet alone, whereas response to GnRH on d 14 appeared to arise as a combination of the effects of both pre- and post-breeding levels of nutrition. The inability of heifers maintained on a high plane of nutrition after breeding to overcome the detrimental effects of undernutrition prior to breeding was unexpected and warrants further investigation.

# Implications

Plane of nutrition prior to and after breeding alters pituitary sensitivity to GnRH during the early stages of the postpartum interval. Maintaining heifers on a low plane of nutrition after breeding reduces pituitary responsiveness to exogenous GnRH on d 3 whereas feeding a high plane of nutrition both prior to and after breeding enhances LH response to GnRH on day 14 postpartum. The negative effects of feeding a low plane of nutrition prior to breeding on GnRH-induced LH release on d 14 were not overcome by increasing the level of nutrition after breeding, suggesting that pre-breeding level of nutrition can influence hypothalamic-pituitary function in postpartum beef heifers.

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